

**Investigating the Molecular Basis of Cold
Temperature and High Pressure Adapted
Growth in *Photobacterium profundum* SS9**

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Declaration

I hereby declare that all work presented in this thesis is my own except where otherwise stated.

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Acknowledgments

I would firstly like to thanks my supervisors, Gail Ferguson and Lindsay Sawyer. Thank you for the opportunity and for your invaluable advice and support.

I also wish to thank all the current and previous lab members from our lab and the neighbouring ones. Without all of you, I have no doubt that the past 4 years would have been more difficult and significantly less fun 😊

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Dedicated to Mum and Dad

Science is a match that man has just got alight. He thought he was in a room – in moments of devotion, a temple – and that his light would be reflected from and display walls inscribed with wonderful secrets and pillars carved with philosophical systems wrought into harmony. It is a curious sensation, now that the preliminary splutter is over and the flame burns up clear, to see his hands lit and just a glimpse of himself and the patch he stands on visible, and around him, in place of all that human comfort and beauty he anticipated – darkness still.

(H. G. Wells, 'The Rediscovery of the Unique', *Fortnightly Review* n.s 50 (1891), p.111.

Table of contents

Declaration	2
Acknowledgments	3
Table of contents	i
List of abbreviations	vi
Abstract	viii
1. Introduction	1
1.1 The diversity of marine microorganisms	1
1.2 Extremophiles	2
1.2.1 High pressure loving bacteria, piezophiles	4
1.2.2 Cold temperature loving bacteria, psychrophiles	6
1.3 The effects of high pressure and cold temperatures on non-pressure adapted bacteria	8
1.4 <i>Photobacterium profundum</i>	11
1.4.1 <i>P. profundum</i> SS9	11
1.4.2 Proteins important for high pressure and cold temperature adaptation	12
1.4.3 Differential gene expression at high pressure, microarray analysis	15
1.4.4 Other <i>P. profundum</i> strains	16
1.4.5 Outer membrane fatty acid composition	18
1.4.6 Transposon mutant screen of <i>P. profundum</i> SS9	18
1.4.7 The <i>P. profundum</i> SS9 putative O-antigen ligase mutant, FL26	21
1.4.8 LPS transport and the role of O-antigen ligase	22
1.4.9 The <i>P. profundum</i> SS9 putative tyrosine protein kinase mutant, FL9	25
1.4.10 The role of Wzc in CPS transport	25
1.5 Aims and objectives	27
2. Materials and Methods	28
2.1 Bacterial plasmids and strains	28
2.2 Antibiotics	30
2.3 Media	30
2.3.1 Luria-Bertani (LB) broth and Agar	30
2.3.2 Marine broth and Agar	30
2.3.3 Low temperature gelling marine agar	31
2.4 Culture conditions	32
2.4.1 <i>Escherichia coli</i>	32
2.4.2 <i>Photobacterium profundum</i> SS9 and 3TCK	32
2.5 Determination of colony forming units	35
2.6 Motility Assay	35
2.7 Basic Biofilm Assay	35
2.8 Microscopy	35
2.8.1 Light Microscopy	35
2.8.2 Lipid staining	36
2.8.3 Flagella staining	36
2.8.4 Transmission Electron Microscopy	37

2.9	LPS extraction and analysis	37
2.9.1	LPS extraction by SDS lysis	37
2.9.2	LPS extraction by hot phenol water	38
2.9.3	LPS extraction by hot phenol water, small scale.....	39
2.9.4	LPS analysis by SDS-PAGE	39
2.9.5	LPS analysis by DOC-PAGE	40
2.9.6	Visualising SDS-PAGE by silver staining	41
2.9.7	Visualising DOC-PAGE by silver staining	42
2.9.8	LPS analysis by HPLC	42
2.10	Cloning and expression	43
2.10.1	Polymerase Chain Reaction (PCR)	43
2.10.2	Agarose gel electrophoresis.....	44
2.10.3	Plasmid purifications	44
2.10.4	Restriction digests	45
2.10.5	Ligation	45
2.10.6	Preparation of competent cells	45
2.10.7	Transformation of <i>E. coli</i>	45
2.10.8	Quick Transformation of <i>Salmonellae</i>	46
2.10.9	Screening transformants by PCR and restriction digest.....	46
2.10.10	Screening transformants by colony blot.....	47
2.10.11	Mating into <i>P. profundum</i> SS9.....	47
2.10.12	Induction and purification	48
2.10.13	Analysis of Protein expression by SDS-PAGE	49
2.10.14	Analysis of proteins by Western blot	50
3.	Results - The effects of temperature and pressure changes on the physiology of <i>P. profundum</i> SS9R	51
3.1	Introduction	51
3.2	Characterisation of growth under different conditions.....	53
3.2.1	Growth of <i>P. profundum</i> SS9R in marine broth.....	53
3.2.2	<i>P. profundum</i> SS9R growth on marine agar.....	58
3.2.3	A method for assessing growth of <i>P. profundum</i> SS9R on solid agar at high pressures	60
3.3	The effects of temperature and pressure on LPS.....	62
3.3.1	SDS-lysis followed by SDS-PAGE does not show significant LPS alterations in response to temperature and pressure	62
3.3.2	Hot phenol water extraction followed by DOC-PAGE shows LPS alterations in response to temperature and pressure	63
3.3.3	Non-piezophilic bacteria do not have the same LPS alterations in response to temperature and pressure.....	65
3.3.4	<i>P. profundum</i> SS9R LPS consists mainly of a glucan.....	67
3.4	The effect of temperature and pressure on the expression of Capsular Polysaccharides (CPS)	76
3.4.1	Investigating the composition of the vacuoles	77
3.5	Discussion	80
3.5.1	<i>P. profundum</i> SS9R growth in liquid media was measured by OD ₆₀₀	80

3.5.2	<i>P. profundum</i> SS9R is a rod-shaped bacterium under optimal growth conditions	80
3.5.3	Growth of <i>P. profundum</i> SS9R on marine agar	81
3.5.4	<i>P. profundum</i> SS9R LPS alters in response to temperature and pressure	82
3.5.5	GC-MS analysis of <i>P. profundum</i> SS9R LPS revealed a glucan	83
3.5.6	TEM analysis of <i>P. profundum</i> SS9R showed the capsule is affected by pressure	84
3.5.7	Analysis of <i>P. profundum</i> SS9R vacuoles/granules	85
3.5.8	Summary	85
4.	Results – Characterisation of the growth and LPS of a putative <i>P. profundum</i> SS9 O-antigen ligase mutant, FL26	87
4.1	Introduction – O-antigen ligase	87
4.2	Investigating the cold temperature sensitivity of FL26 relative to the parent strain, SS9R	90
4.2.1	FL26 displays a cold temperature sensitive phenotype on marine agar ..	90
4.2.2	FL26 does not display a cold temperature sensitive phenotype in marine broth	92
4.2.3	FL26 does not show a high pressure growth alteration using marine agar overlay plates	94
4.3	Analysis of FL26 LPS	96
4.3.1	The LPS profile of FL26 shows alterations that are characteristic of an O-antigen ligase mutant	96
4.3.2	LPS alterations can affect other processes, such as biofilm formation and motility	102
4.4	Other cold temperature sensitive putative polysaccharide biosynthesis mutants share similar phenotypes to FL26	105
4.5	A comparison with the <i>S. Typhimurium</i> LT2 O-antigen ligase mutant, SL3749	111
4.5.1	The loss of O-antigen in <i>S. Typhimurium</i> LT2 does not lead to cold temperature sensitive growth	111
4.5.2	<i>S. Typhimurium</i> LT2 does not alter LPS profile in response to cold temperatures or growth on agar	114
4.5.3	SL3749 has a reduction in swimming motility compared to the parent strain	115
4.6	Complementation of <i>P. profundum</i> SS9R FL26	118
4.6.1	The <i>P. profundum</i> SS9 complementation vector pFL190	118
4.6.3	Expression of pDA01 (pFL190 <i>pbpra0218</i>) will partially complement the LPS profile alteration in FL26	121
4.7	Complementation of <i>S. Typhimurium</i> LT2 SL3749	126
4.7.1	Expression of pDA01 (pFL190 <i>pbpra0218</i>) will not complement the LPS related phenotypes of SL3749	126
4.7.2	Expression of pDA03 (pFL190 <i>S. Typhimurium</i> LT2 O-antigen ligase) will complement the LPS related phenotypes of SL3749	129
4.8	Discussion	133
4.8.1	The <i>P. profundum</i> SS9 gene, <i>pbpra0218</i> , encodes an O-antigen ligase	133

4.8.2	FL26 displays a cold temperature sensitive phenotype on marine agar	134
4.8.3	FL26 displays an LPS profile alteration by SDS-PAGE that can be partially complemented	136
4.8.4	FL26 displays an increased motility compared to the parent strain	137
4.8.5	Cold temperature sensitive LPS biosynthesis mutants of <i>P. profundum</i> SS9 also have an increased motility	138
4.8.6	Summary and conclusions	139
5.	Results – Characterisation of the growth and LPS of a putative <i>P. profundum</i> SS9 tyrosine protein kinase mutant, FL9	141
5.1	Introduction – tyrosine protein kinase, Wzc	141
5.2	Investigating the cold temperature sensitivity of FL9 relative to the parent strain, SS9R	144
5.2.1	FL9 displays a cold temperature sensitive phenotype on marine agar	144
5.2.2	FL9 does not display a cold temperature sensitive phenotype in marine broth	145
5.2.3	FL9 does not show a high pressure growth alteration using marine agar overlay plates	147
5.3	Analysis of FL9 LPS	149
5.3.1	The LPS profile of FL9 shows alterations that are characteristic of a <i>wzc</i> mutant	149
5.3.2	FL9 does not have an alteration in biofilm or motility compared to the parent strain	152
5.3.3	FL9 has a reduction in capsule characteristic of Wzc mutants	153
5.4	A comparison with the <i>Escherichia coli wzc</i> mutant, CWG285	155
5.4.1	A mutation in <i>wzc</i> in <i>E. coli</i> does not lead to cold temperature sensitive growth	155
5.4.2	CWG285 has an increase in swimming motility compared to the parent strain	158
5.5	Complementation of <i>P. profundum</i> SS9R FL9	159
5.5.1	Expression of pDA02 (pFL190 <i>pbpra2686</i>) will partially complement the LPS profile alteration in FL9	159
5.6	Discussion	163
5.6.1	The <i>P. profundum</i> SS9 gene, <i>pbpra2686</i> , encodes a tyrosine protein kinase, Wzc	163
5.6.2	FL9 displays a cold temperature sensitive phenotype on marine agar	164
5.6.3	The <i>E. coli wzc</i> mutant, CWG285, was found to have an increased motility	165
5.6.4	Summary and conclusions	166
6.	Concluding remarks	167
	Appendix A – Cloning of <i>P. profundum</i> SS9 O-antigen ligase, <i>pbpra0218</i>	171
	Appendix B – Cloning of <i>S. Typhimurium</i> O-antigen ligase	182
	Appendix C – Cloning of <i>P. profundum</i> SS9 tyrosine protein kinase, <i>pbpra2686</i>	185
	Appendix D– Cloning, expression and purification of <i>P. profundum</i> SS9 Ldh	196
D.1	Introduction	196

D.2	Cloning and expression of <i>P. profundum</i> SS9 Ldh in <i>E. coli</i> M15 pREP4 pQE30.....	197
D.3	Purification of <i>P. profundum</i> SS9 Ldh.....	204
D.4	Discussion	214
D.4.1	Cloning and screening of pbpra1210.....	214
D.4.2	Expression and purification of <i>P. profundum</i> SS9 Ldh.....	214
D.4.3	Conclusions	215
References	216

List of abbreviations

%	percent
°C	degrees celcius
µg	microgram
µl	microlitre
APS	Ammonium persulphate
β-HB	Betahydroxybutyrate
BLAST	Basic local alignment search tool
bp	base pairs of DNA
CPS	Capsular polysaccharide
DNA	Deoxyribonucleic acid
DOC	Deoxycholic acid
EPS	Exopolysaccharide
FabF	β-keto-acyl-synthase II
g	gram
GC-MS	Gas Chromatography Mass Spectroscopy
HPLC	High performance liquid chromatography
IPTG	Isopropylthiogalactopyranoside
kDa	kilodaltons, refers to mass of protein
kb	kilobase pairs of DNA
l	Litre
Ldh	Lactate dehydrogenase
LTGMA	Low temperature gelling marine agar
LPS	Lipopolysaccharide
M	Molar
Mdh	Malate dehydrogenase
mg	milligram
ml	millilitre
mM	millimole

MUFA	Monounsaturated fatty acids
NMR	Nuclear magnetic resonance
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PHB	Polyhydroxybutyrate
PUFA	Polyunsaturated fatty acids
RBS	Ribosome binding site
rpm	Revolutions per minute
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
TEM	Transmission electron microscopy
TEMED	Tetramethylethylenediamine
UFA	Unsaturated fatty acids
VBNC	Viable but non-culturable
% v/v	Percentage volume per volume
% w/v	Percentage weight per volume
% w/w	Percentage weight per weight
X-Gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

Abstract

Photobacterium profundum SS9 is a γ -proteobacterium which grows optimally at 15°C and 28 MPa (a psychrophilic piezophile) and can grow over a range of temperatures (2-20°C) and pressures (0.1-90 MPa). Previous research had demonstrated that *P. profundum* SS9 adapts its membrane proteins and phospholipids in response to growth conditions. In this study, methodology was developed for growing *P. profundum* SS9 under cold temperatures and high pressures in both liquid and solid cultures. The effect of changing growth conditions on cell envelope polysaccharides was then investigated. The lipopolysaccharide (LPS) profile of a rifampicin resistant *P. profundum* SS9 derivative, SS9R, was shown to change at 0.1 MPa with respect to temperature and at 15°C with respect to pressure. Compositional analysis showed that the LPS was almost entirely composed of glucose. This provides evidence that, under these conditions, the major polysaccharide produced by *P. profundum* SS9 is a glucan.

Two putative polysaccharide mutants, FL26 & FL9, were previously isolated from a screen for cold-sensitive mutants of *P. profundum* SS9R. Both mutants displayed an increased sensitivity to cold temperatures on solid medium and were unaffected in their growth at high pressure. FL26 was found to exhibit an LPS alteration similar to previously published O-antigen ligase mutants, providing evidence that this mutant is likely to lack O-antigen ligase. Interestingly, FL26 was also shown to have a reduced ability to form biofilms and had increased swimming motility. This suggests that there are a number of changes which occur in FL26 in the absence of O-antigen. FL9 was found to have an altered LPS and capsular polysaccharide (CPS), similar to an *E. coli* *wzc* mutant. In *E. coli*, Wzc is involved in the polymerisation and transport of CPS, disruption of which can also lead to LPS alterations. The LPS and CPS alterations may lead to the cold-sensitivity phenotype, either individually or in combination.

In conclusion, alterations in the cell envelope polysaccharides were shown to affect cold temperature sensitivity on solid agar. Cold-sensitivity is most likely directly related to the LPS alterations and stability of the membrane under cold temperatures. Exopolysaccharides (EPS) have previously been shown to affect desiccation and freeze-thaw resistance, making it is possible that the CPS plays a similar role in this case.

1. Introduction

1.1 The diversity of marine microorganisms

70% of the Earth's surface is covered by Oceans, which have an average depth of 3.8 km, an average pressure of 38 MPa ($0.1 \text{ MPa} = 1 \text{ atm} = 1 \text{ bar}$) and an average temperature of 2°C . The deepest part of the Oceans is the Mariana Trench in the Atlantic Ocean, reaching over 10 km deep with a maximum pressure of 110 MPa (Yayanos *et al.*, 1981). Even in such extreme environments with cold temperatures and high pressures, bacteria have been isolated (Kato *et al.*, 1998; Yayanos *et al.*, 1981). However, estimates of microbial numbers are still unreliable: PCR libraries, metagenomic libraries, growth and direct microscopic observation are capable of misrepresenting the actual numbers of microorganisms present in a population (Cottrell *et al.*, 2005; Jannasch and Jones, 1959). For example, by comparing the results from counting colony growth and direct microscopic observation, the estimates were found to be at least 0.1% of the actual microscopic life present (Staley and Konopka, 1985). Numerous studies have explored the microbial communities of the deep sea by creating metagenomic libraries (DeLong *et al.*, 2006). As mentioned above, it is difficult to ascertain whether these libraries accurately represent the diversity of the deep sea, however the isolation and culturing of such bacterial species is inherently difficult. This is partially due to the great variation in nutrient abundance, such as dissolved carbon, phosphate and oxygen in the various depths of the oceans (Benner *et al.*, 1992; DeLong *et al.*, 2006; Druffel *et al.*, 1992). The isolation and culturing of environmental bacteria can therefore be difficult due to extremely slow growth rates and nutrient requirements that are difficult to replicate in the laboratory. This can also be attributed in part to the viable but nonculturable (VBNC) state (Oliver, 2005). Bacteria in a VBNC state often will not grow and are difficult to detect by metabolic assays. For this reason our understanding of the physiology of bacteria from extreme environments has been limited to those that are culturable.

1.2 Extremophiles

Extremophiles are characterised as microorganisms that are capable of optimal growth in extreme conditions of temperatures, pH, salt concentrations, pressures, etc. These microorganisms have evolved a variety of novel mechanisms for coping with such extreme conditions. A good example is *Pyrolobus fumarii*, which was isolated at a depth 3.6 km from a hydrothermal in the midatlantic ridge (Blöchl *et al.*, 1997). *P. fumarii* will grow only in the high temperature range 90-113°C and is currently the microorganism with the highest recorded growth temperature. *P. fumarii* was also capable of surviving a 1 hour autoclaving cycle at 121°C and 25 MPa. Due to its membrane composition and 16sRNA sequence, *P. fumarii* was found to be a member of the archaea. Archaea are a group of prokaryotes they are distinctly different to bacteria and belong to a separate phylum. While archaea are not exclusively extremophiles, some of the most interesting examples of extreme growth do belong to the archaea. There are several distinct differences between archaea and bacteria, one of which is the structure and composition of the membranes. Bacteria have a phospholipid bilayer containing glycerol-ester-lipids whereas archaeal membranes contain glycerol-ether-lipids (De Rosa *et al.*, 1986) (Figure 1). Ether bonds are more resistant to oxidation and high temperatures than ester bonds, which may explain the ability of some archaea to survive in extremes of temperature and pH (Albers *et al.*, 2000). In some archaea the phospholipid bilayer has been substituted for a monolayer in which the lipid tails of the phospholipids are fused together to form a dipolar phospholipid. Examples include *Ferroplasma acidarmarinus*, which grows optimally in pH 1.2 (Macalady *et al.*, 2004) and *Methanopyrus kandleri*, a hyperthermophilic methanogen which has an optimal growth temperature of 98°C (Kurr *et al.*, 1991). These dipolar phospholipids make the membrane more rigid and more able to survive highly acidic pH and high temperatures (Elferink *et al.*, 1992). The lipid tails of the phospholipids are also different in archaea compared to bacteria. In bacteria these consist of mostly un-branched fatty acids without any side chains. Archaeal lipid tails are based on isoprenoid, and can contain cyclohexane and cyclopropanes (Damste *et al.*, 2002) (Figure 1). Branched lipid chains such as those have been shown to help maintain the

integrity of membranes under temperature extremes (Albers *et al.*, 2000; Koga and Morii, 2005).

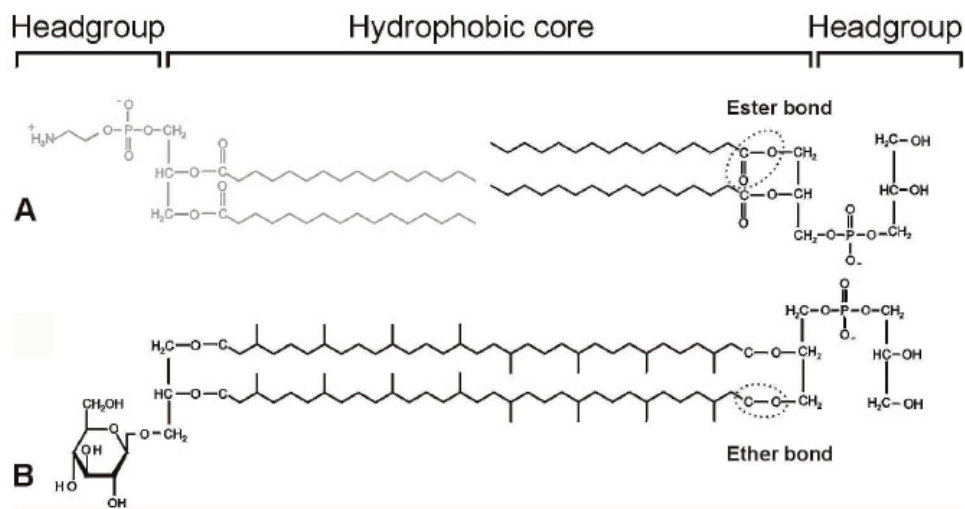


Figure 1: The structure of bacterial and archaeal lipids

A) Lipids forming a bilayer in *Escherichia coli*. The connection of the acyl chain with the headgroup is ester. In the above example the acyl chain is straight, but branched groups are not uncommon. B) Monolayer of lipids in the thermoacidophilic archaea *Thermoplasma acidophilum*. The connection of the phytanyl chain with the headgroup is ether. The phytanyl chain contains isoprenoid-like branches. [Figure from (Albers *et al.*, 2000)].

Like bacteria, archaea have been shown to alter their membrane composition in response to temperature (discussed later). In general, archaea have been demonstrated to increase the numbers of cyclopropane rings as temperature increases (Schouten *et al.*, 2002). *Methanococcoides burtonii* is an archaea originally isolated from Antarctica at 1-2°C. This archaea has been shown to have an increased proportion of unsaturated fatty acids in membrane phospholipids at 4°C compared to 23°C (Nichols *et al.*, 2004).

The large number of extremophiles and the diversity of their adaptations allows for the possibility of using these adaptations for our own benefit (Table 1). Enzymes from extreme environments have already been utilised, with one of the most well known examples being the DNA polymerase from *Thermus aquaticus*. *T. aquaticus* is a thermophilic bacterium capable of growth in temperatures from 50-80°C. The DNA

polymerase was found to have optimal activity at 80°C and as such was ideal for use in PCR, which requires numerous cycles at high temperatures (Chien *et al.*, 1976).

Cold-active lipases can be used in biological washing powders to enable lower washing temperatures. Cold-active enzymes are also useful in the biotechnology industry, as the enzymes are thermally labile and therefore easy to rapidly thermally inactivate. In fields where high temperatures are undesirable, such as the food industry, inactivation of enzymes at lower temperatures maintains the quality of temperature-sensitive food products.

Table 1: Examples of useful enzymes from extremophiles

Type	Growth characteristics	Enzymes	Applications
Thermophiles	Temp >80°C (hyperthermophile) and 60–80°C (thermophile)	Proteases	Detergents, hydrolysis in food and feed, brewing, baking
		Glycosyl hydrolases (e.g. amylases, pullulanase, glucoamylases, glucosidases, cellulases, xylanases)	Starch, cellulose, chitin, pectin processing, textiles
		Chitinases	Chitin modification for food and health products
		Xylanases	Paper bleaching
		Lipases, esterases	Detergents, stereo-specific reactions
		DNA polymerases	(e.g. <i>trans</i> -esterification, organic biosynthesis)
Psychrophiles	Temp <15°C	Dehydrogenases	Molecular biology (e.g. PCR)
		Proteases	Oxidation reactions
		Amylases	Detergents, food applications (e.g. dairy products)
		Cellulases	Detergents and bakery
		Dehydrogenases	Detergents, feed and textiles
		Lipases	Biosensors
Halophiles	High salt, (e.g. 2–5 M NaCl)	Proteases	Detergents, food and cosmetics
Alkaliphiles	pH >9	Dehydrogenases	Peptide synthesis
		Proteases, cellulases	Biocatalysis in organic media
Acidophiles	pH <2–3	Amylases, glucoamylases	Detergents, food and feed
		Proteases, cellulases	Starch processing
Piezophiles	Pressure-loving; up to 130 MPa	Oxidases	Feed component
		To be defined	Desulfurization of coal
			Food processing and antibiotic production

[Table taken from (van den Burg, 2003)]

1.2.1 High pressure loving bacteria, piezophiles

Little is known or understood about the deep sea, especially details of how microorganisms are capable of growth under such extreme pressures. With regard to growth under elevated pressures, organisms can be divided into 3 classes: 1) those that grow optimally under pressures between >0.1 MPa and <60 MPa are piezophiles (Yayanos, 1995) (Figure 2), 2) organisms that are capable of growth under high

pressure, but not optimally, are piezotolerant, 3) and those which have reduced growth at high pressures are piezosensitive.

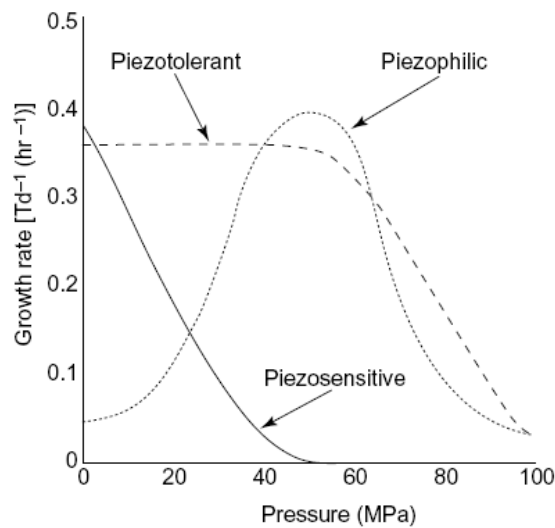


Figure 2: The different growth responses to high pressures

Piezosensitive bacteria have a reduced growth rate with elevated pressures. Piezotolerant bacteria are capable of growth over elevated pressures, but not optimal growth. Piezophilic bacteria show optimal growth at elevated pressure. [Figure from (Abe and Horikoshi, 2001)].

In a piezotolerant bacteria, the levels of unsaturated fatty acids have been found to increase with elevated pressure (DeLong and Yayanos, 1985; DeLong and Yayanos, 1986; Kamimura *et al.*, 1992). One piezotolerant bacterium RS103 which can grow in pressures up to 60 MPa, contains majorly branched fatty acids at atmospheric pressure, although upon increasing pressure a greater proportion of unsaturated fatty acids C17:1 and C18:1 were incorporated (Kamimura *et al.*, 1993). Upon decreasing the growth temperature the proportion of unsaturated fatty acids also increased, mostly due to greater incorporation of C16:1 (Kamimura *et al.*, 1992). Unsaturated fatty acids are a common feature of piezophilic bacteria, with numerous piezophilic *Shewanella* strains having been demonstrated to increase the proportion of the polyunsaturated fatty acids EPA (C20:5) and DHA (C22:6) with respect to an increase in pressure (DeLong *et al.*, 1997; Kato *et al.*, 1998; Nogi *et al.*, 1998a).

An example of an adaptation other than in the membrane composition is in *Shewanella violacea*, a piezophilic bacterium that grows optimally at 30 MPa and 8°C (Kato *et al.*, 1995; Kato *et al.*, 1998). The purified RNA polymerase from *S. violacea* was compared to the RNA polymerase from *E. coli* for stability under high pressures (Kawano *et al.*, 2004). After a 30 minute treatment at 100 MPa the *E. coli* RNA polymerase activity was reduced to 60% whereas the *S. violacea* RNA polymerase maintained full activity.

1.2.2 Cold temperature loving bacteria, psychrophiles

From the piezophiles discussed in the previous section, it is clear that bacteria often have several requirements/adaptations such as high pressure (piezophilic) and cold temperature (psychrophilic). It is also interesting to note that all culturable psychrophilic piezophiles have been found to belong to one of 5 phylogenetic groups, either *Shewanella*, *Colwellia*, *Moritella*, *Photobacterium* or the CNPT-3 group (DeLong *et al.*, 1997; Radjasa, 2004).

Psychrophiles have evolved to grow optimally in temperatures <20°C (Morita, 1975). One extreme example is *Psychromonas ingrahamii*, a marine psychrophile capable of growth at -12°C, albeit with a doubling time of 240 hours (Breezee *et al.*, 2004; Riley *et al.*, 2008). *P. ingrahamii* grows optimally at 5°C, doubling every 12 hours (Breezee *et al.*, 2004).

Bacteria have been shown to alter the cell envelope composition in response to different environmental conditions. The cell envelope of gram negative bacteria contains an inner membrane which consists of a phospholipid bilayer (Figure 3). The outer membrane consists of a phospholipid layer on the periplasmic face and a lipopolysaccharide (LPS) layer on the extracytoplasmic face. The outside of the cell is often covered with a capsular polysaccharide (CPS) and the cell can extrude an exopolysaccharide (EPS) into the surrounding environment. The general structure of a LPS molecule consists of a fatty acid tail (lipid A) attached to a sugar core and a polysaccharide chain (O-antigen).

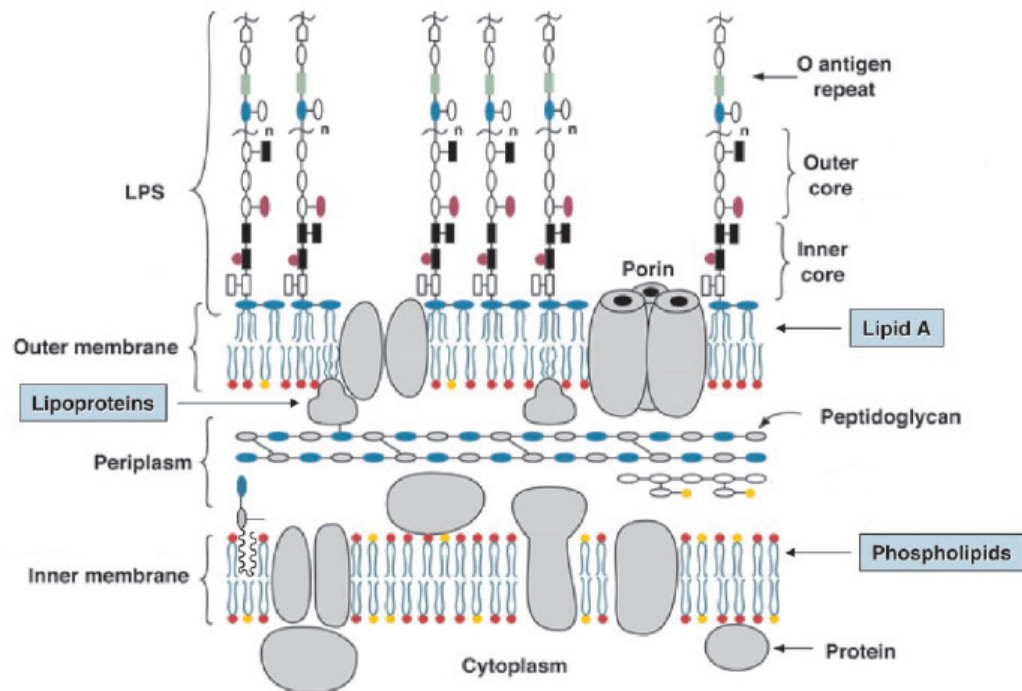


Figure 3: Schematic representation of the gram-negative cell envelope

The inner membrane is composed mainly of a phospholipid bilayer. The periplasmic face of the outer membrane consists of another phospholipid layer, while the extracytoplasmic face of the outer membrane consists of lipopolysaccharide (LPS). LPS is composed of Lipid-A, a sugar core and O-antigen. [Figure from (Raetz and Whitfield, 2002)].

Psychrophilic bacteria have been demonstrated to alter their LPS and EPS composition in response to cold temperatures. One of the most studied psychrophiles in terms of LPS composition is *Pseudomonas syringae*. The LPS of *P. syringae* was demonstrated to have a reduced amount of phosphorylation (Ray *et al.*, 1994) at 4°C compared to 22°C. The significance of the phosphorylation state of the LPS has yet to be fully investigated, but has been hypothesised to affect the permeability of the membrane (Ray *et al.*, 1994). The outer membrane was also shown to have an increased fluidity at 4°C compared to 22°C, and that this is related to an increased proportion of hydroxy fatty acids in the LPS at 4°C (Kumar *et al.*, 2002).

Examples of cold adaptation in protein activities include a Malate dehydrogenase (Mdh) from the psychrophile *Vibrio marinus*, which was found to be stable between 0 and 14°C (measured on whole cells), above which the enzyme lost activity with increasing temperature (Langridge and Morita, 1966). *Photobacterium lipolyticum* M37 is a bacterium that can grow at temperatures as low as 1°C and optimally at 27°C (Yoon *et al.*, 2005). A cold-adapted lipase from *P. lipolyticum* was shown to have a functional temperature range of 2 to 50°C (Ryu *et al.*, 2005). While the lipase nevertheless demonstrated high activity at 2°C (75% of maximum), the optimum activity was at 25°C. Interestingly, the lipase from *P. lipolyticum* does not share significant sequence homology with published bacterial lipases, but does share homology with lipases from filamentous fungi, specifically in the active site. Mdh's and Lactate dehydrogenases (Ldh) are useful enzymes to characterise from novel bacteria because the function of these enzymes has been thoroughly investigated and the biochemical properties and structure of many such enzymes are known.

1.3 The effects of high pressure and cold temperatures on non-pressure adapted bacteria

Pressure affects bacteria in a number of different ways, for example, *E. coli* DNA synthesis is inhibited at 50 MPa, protein synthesis is inhibited at 58 MPa and RNA synthesis is inhibited at 77 MPa (Yayanos and Pollard, 1969). Under these pressures the RNA polymerase remains associated with the DNA template, and will resume polymerisation when the pressure is reduced, so-called reversible stalling (Erijman and Clegg, 1998). Factors which influence synthesis include the effects of pressure on ribosome conformation and protein structure. Ribosomes exist in equilibrium of associated and dissociated states. Under high pressures *E. coli* ribosomes have been shown to favour the dissociated state, and when the pressure was increased from 10 to 20 MPa the amount of time ribosomes spent dissociated was effectively doubled (Gross and Jaenicke, 1990; Niven *et al.*, 1999; Schulz *et al.*, 1976). This has a negative effect on the initiation of transcription. Pressure also directly affects the structure of proteins,

causing denaturation. In general, primary and secondary protein structures are more resistant to high pressures than tertiary, quaternary and multimeric structures (Graumann and Marahiel, 1996; Heremans, 1982; Silva and Weber, 1993).

Apart from macromolecular synthesis, membranes are also affected by elevated pressures. This has been documented in various piezophilic bacteria, however piezo-sensitive bacteria, such as *E. coli*, have not been shown to alter membrane composition in response to elevated pressure (Allen and Bartlett, 2000). Models have shown that high pressures induce a tighter packing of membrane lipids from non-pressure adapted bacteria, increasing viscosity of the membrane and it becomes more gel-like (Denich *et al.*, 2003). This results in loss of membrane function such as a fatal increase in membrane permeability (Ganzle and Vogel, 2001).

Many bacteria have been shown to alter their composition of fatty acids in response to temperature changes. For example, *E. coli* is known to increase the relative proportions of the unsaturated fatty acids C16:1, C18:1 and C20:1 when grown at 15°C compared to 43°C (Cronan, 1975). This corresponds with a reciprocal decrease in the amount of saturated fatty acids. These modifications to membrane lipids bring about homeoviscous adaptation, a mechanism which maintains the fluidity of the membrane under different temperatures (Sinensky, 1974).

Various systems have evolved in bacteria to cope with water stress, most of which consist of the accumulation of molecules to balance the internal osmotic pressure (compatible solutes). Such molecules include inorganic ions (Na^+ and K^+) amino acids (proline and glutamine) and organic osmolytes (Betaine and TMAO, etc.) (Yancey *et al.*, 1982). For example, in response to growth in high concentrations of NaCl, *E. coli* will synthesise or import more proline and glycine betaine. The effects of osmolytes and high pressure on protein-DNA complexes was studied using the restriction enzyme *EcoRI*, which has a high specificity for the DNA sequence GAATTC under standard circumstances. It was found that high concentrations of osmolytes decreased the specificity of *EcoRI* therefore increasing its star activity (digestion at sites other than GAATTC) (Robinson and Sligar, 1994). By increasing the hydrostatic pressure the effect of osmolytes was reversed, returning the specificity of *EcoRI*.

In bacteria that are not adapted to high pressures, such as *E. coli*, exposure to high pressures induces a unique stress response that has a protein expression profile with similarities to both heat and cold shock (Welch *et al.*, 1993). One explanation for this is that high pressures and cold temperatures exert similar physical effects on biological membranes, which decrease overall membrane fluidity (Chong *et al.*, 1981; Macdonald, 1984). Temperature and pressure share such a relationship that the membrane of a deep sea bacterium at 2°C (100 MPa) is equivalent to an identical membrane at -18°C (0.1 MPa) (Bartlett, 1999).

Cold shock involves the expression of proteins (Csps) that enhance cellular processes such as protein folding and maintaining RNA/DNA conformation at cold temperatures. In *E. coli*, a temperature downshift causes a temporary inhibition of most protein synthesis, the resulting growth lag is called the acclimation phase. During this phase Csp's are induced, there are two classes of Csp's (Thieringer *et al.*, 1998). Class I are expressed at a very low level at 37°C and are dramatically increased under cold shock. Class II are present at a steady state level at 37°C and are further induced by only a few fold in cold shock. A group of structurally related Class I Csp's in *E. coli*, called the CspA family, contain the major cold shock proteins responsible for the stabilisation of RNA and DNA at cold temperatures maintaining transcription and translational processes (Phadtare *et al.*, 1999). Most *Enterobacteriaceae* have a family of proteins homologous to CspA, however not all have a role in cold adaptation. In psychrophiles, CspA-like proteins called Cold acclimation proteins (Caps) are present at a steady level and increase upon a shift to lower temperatures (Whyte and Inniss, 1992). It has been proposed that the persistent level of Caps in psychrophiles could be one of the key factors in cold temperature adaptation.

1.4 *Photobacterium profundum*

1.4.1 *P. profundum* SS9

Photobacterium profundum SS9 is a psychrophilic and piezophilic bacterium, that is capable of growth over a range of temperature (2-20°C) and pressures (0.1-90 MPa), while growing optimally at 15°C and 28 MPa (DeLong, 1986). *P. profundum* SS9 was originally isolated from the Sulu sea at a depth of 9 km (2°C) and is a gram-negative, γ -proteobacterium and a member of the *Vibrionaceae*. Since its isolation, the genome has been fully sequenced and like other members of the *Vibrionaceae*, *P. profundum* SS9 has two circular chromosomes (4.1 and 2.2 Mb respectively) and also contains an 80 kb plasmid (Trucksis *et al.*, 1998; Vezzi *et al.*, 2005). The plasmid has contains no essential genes and can be lost in the laboratory with no obvious phenotypic changes (Campanaro *et al.*, 2005). Due to its wide range of growth conditions and genetic tractability, *P. profundum* SS9 is used as a model piezophile.

It is interesting to note that the *P. profundum* SS9 genome has also been shown to contain 15 ribosomal RNA genes, making it the equal highest recorded in bacteria (Rainey *et al.*, 1996; Vezzi *et al.*, 2005). The numbers of rRNA operons has been linked to both growth rate and the role of adapting to the environment (Klappenbach *et al.*, 2000; Pruss *et al.*, 1999). A study has also demonstrated a link between rRNA GC content and growth temperature from mesophilic and hyperthermophilic bacteria (Wang *et al.*, 2006). 16S rRNA from bacteria which grew optimally at higher temperatures were found to have an increased GC content and increased sequence length. However this study did not take into account psychrophiles or piezophiles. A later study has demonstrated a relatedness between the structure of rRNA from piezophilic bacteria (Lauro *et al.*, 2007). In general, piezophilic bacteria were shown to have extended regions in the rRNA that was not present in non-pressure adapted bacteria. While the sequences of the extensions were hypervariable it was shown that these extensions were almost exclusively a feature of piezophiles

As mentioned above, bacteria have been shown to accumulate inorganic ions, amino acids and organic compounds to equalize osmotic pressure (Yancey *et al.*, 1982). *P. profundum* SS9 has also been shown to perform a similar adaptation in relation to growth phase, cold temperatures and high pressures (Martin *et al.*, 2002). NMR analyses found that the accumulation of alanine, betaine, glutamine and β -hydroxybutyrate (β -HB) all changed with respect to growth phase and when in the presence of high salt concentrations. Importantly, β -HB was also shown to be accumulated in high amounts when *P. profundum* SS9 was grown at cold temperatures or high pressures.

1.4.2 Proteins important for high pressure and cold temperature adaptation

A large amount of work has been performed on the expression and activity of individual proteins of *P. profundum* SS9. The expression of the membrane proteins OmpH and OmpL were found to respond inversely to pressure, with OmpH abundance increasing with increased pressure and OmpL abundance increasing with decreased pressure (Figure 4) (Bartlett *et al.*, 1989; Bartlett and Chi, 1994; Bartlett *et al.*, 1993; Bartlett and Welch, 1995; Chi and Bartlett, 1993; Welch and Bartlett, 1996). While not essential for high pressure-adapted growth, OmpH has been theorised to be necessary for nutrient acquisition in the deep sea (Bartlett and Chi, 1994), since nutrients are known to be less abundant at greater depths (Benner *et al.*, 1992; Druffel *et al.*, 1992).

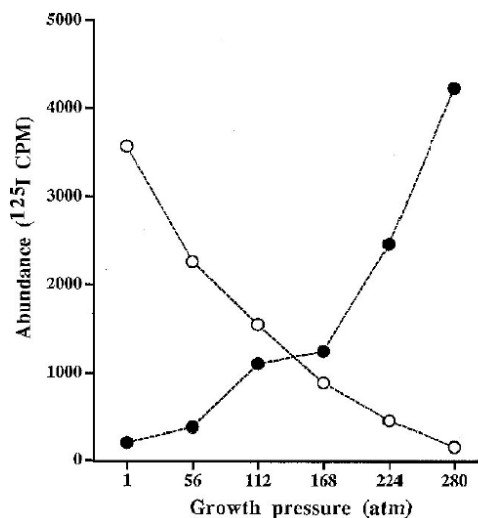


Figure 4: Relative abundance of OmpL and OmpH in *P. profundum* SS9 under different pressures

Anti-OmpH/L antiserum and 125 I-iodine-labelled protein A were used in conjunction with Western blotting and gamma counting to measure the abundance of OmpL (\circ) and OmpH (\bullet). [Figure from (Welch and Bartlett, 1996)].

In order to identify genes involved in the regulation of pressure sensing, a random transposon mutagenesis was performed on *P. profundum* SS9 and mutants were screened by western blot for a lack of OmpL, a protein whose abundance is known to be affected by pressure (Welch and Bartlett, 1996). Southern hybridisation revealed that the transposon insertion was in *toxR*, a gene encoding part of the ToxRS two component regulator (Bidle and Bartlett, 2001; Welch and Bartlett, 1998). ToxRS is known to be an important regulator in *Vibrio cholerae*, modulating protein expression in relation to various environmental cues including temperature (Miller *et al.*, 1989; Parsot and Mekalanos, 1990; Parsot *et al.*, 1991). The hypothesis to explain the role of ToxRS in pressure sensing in *P. profundum* SS9 was that pressure-derived alterations in the inner membrane affect the conformation of ToxR and ToxS and therefore their effect on signal transduction (Figure 5) (Abe *et al.*, 1999).

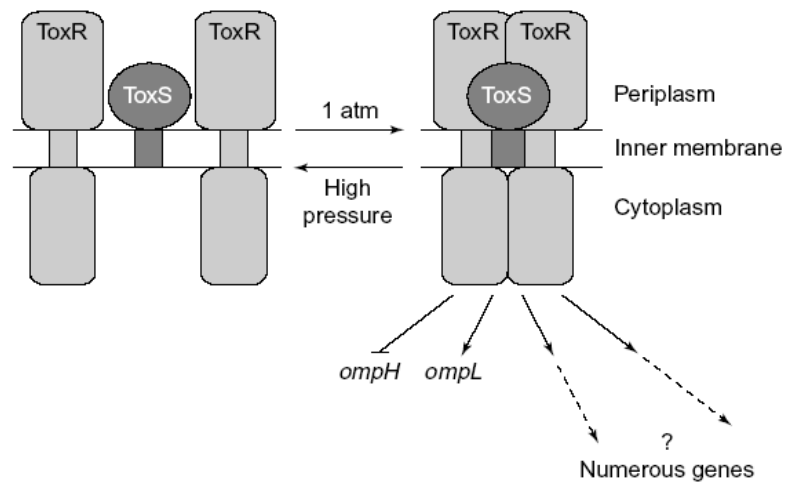


Figure 5: Hypothesised model for the role that ToxRS proteins play in pressure sensing in *P. profundum* SS9

At 0.1 MPa, ToxR and S are associated and activate *ompL* expression and repress *ompH* expression. At elevated pressures ToxR and S undergo a conformational change that prevents *ompL* expression and *ompH* repression. [Figure from (Abe *et al.*, 1999)].

Other proteins of potential importance were σ^E and RecD. The alternative sigma factor, σ^E , is known to be involved in responding to cell extracytoplasmic stresses and misfolded proteins in the outer membrane (Ades *et al.*, 1999). σ^E has also been demonstrated to be necessary for psychrophilic and piezophilic growth in *P. profundum* SS9 (Chi and Bartlett, 1995). A disruption in *rpoE* prevents growth of *P. profundum* SS9 at 5°C (0.1 MPa) and displayed reduced growth at pressure 28 MPa (9°C). The disruption in *rpoE* also led to loss of OmpH at atmospheric pressure, and was in fact the initial basis used for screening for pressure regulatory mutants. Interestingly, chemically derived changes in the Lipid A structure has also been shown to activate the σ^E dependant response in *E. coli* (Tam and Missiakas, 2005).

RecD was found to be required for piezophilic growth in *P. profundum* SS9 (Bidle and Bartlett, 1999). RecD is part of the RecBCD complex and is required for the homologous recombination pathway, repairing double-stranded DNA breaks. RecD mutants are hyperrecombinogenic as a result (Biek and Cohen, 1986; Myers and Stahl,

1994). When *recD* from *P. profundum* SS9 was placed into an *E. coli* mutant that lacks RecD, the growth at elevated pressure was not significantly affected. However the *P. profundum* SS9 *recD* did affect the *E. coli* cell morphology. *E. coli* has been well characterised to filament when grown at elevated pressures (Zobell and Cobet, 1962), but by expression of the *P. profundum* RecD in an *E. coli* *recD*- mutant, the filamentation phenotype was reversed and the cells were rod shaped at elevated pressures (Bidle and Bartlett, 1999).

An array of other proteins and mechanisms have also shown to be important for high pressure and cold temperature adapted growth through the creation of a random transposon mutant library (see section 1.4.6) (Lauro *et al.*, 2008).

1.4.3 Differential gene expression at high pressure, microarray analysis

To investigate the activity of the *P. profundum* SS9 genome under different environmental conditions, microarray analysis was performed on cultures grown at 0.1, 28 and 45 MPa (15°C) and 4 and 15°C (0.1 MPa) (Vezzi *et al.*, 2005) (Figure 6). The high pressure analysis revealed 260 genes grouped into 14 different classes that had differential expression at high pressure compared to 0.1 MPa. This demonstrated that genes involved in protein folding, DNA repair, ion and amino acid transport and glycolysis were up-regulated at 0.1 MPa. Protein folding, DNA repair and glycolysis are all known to be upregulated in bacteria under stressful conditions and therefore being stressed at 0.1 MPa compared to 28 MPa would indicate that *P. profundum* SS9 is a true piezophile. Despite growing optimally at 28 MPa (15°C), these stress response genes at not upregulated at 45 MPa.

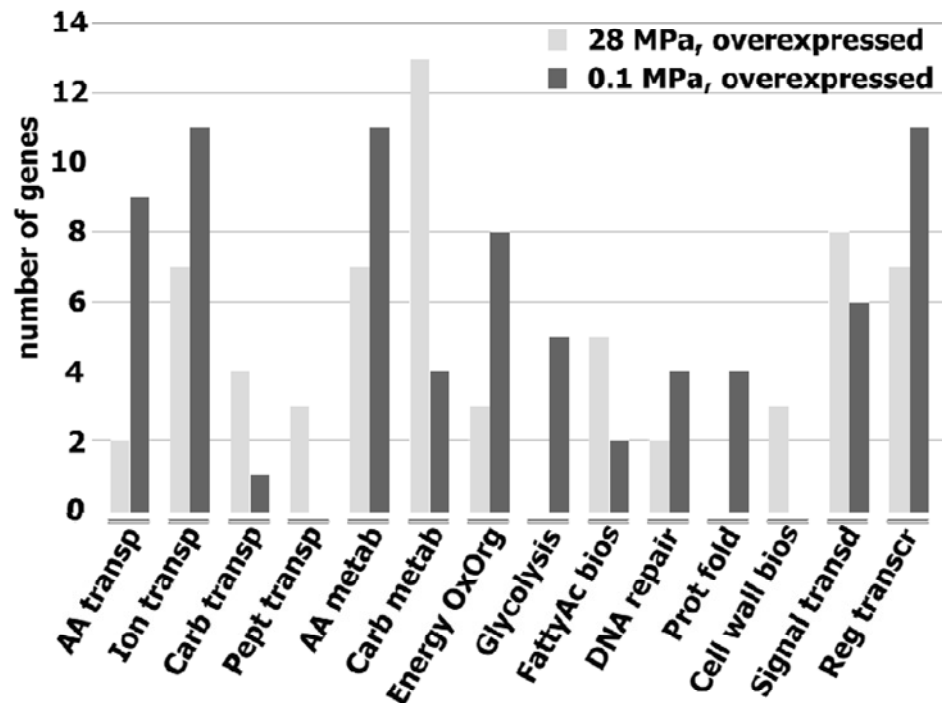


Figure 6: Summary of differentially expressed genes at 28 and 0.1 MPa in *P. profundum* SS9

AA transp (Amino acid transport), Ion transp (Inorganic Ion transport), Carb transp (carbohydrate transport), Pept transp (Peptide transport), AA metab (Amino acid metabolism), Carb metab (Carbohydrate metabolism), Energy OxOrg (Energy production conversion), FattyAc bios (Fatty acid biosynthesis), Prot fold (protein folding), Cell wall bios (Cell wall biosynthesis), Signal transd (Signal transduction), Reg transcr (Regulation of transcription). [Image taken from (VeZZi *et al.*, 2005) supplementary material].

1.4.4 Other *P. profundum* strains

As well as SS9, there are currently two other *P. profundum* strains which have been isolated, 3TCK and DSJ4. *P. profundum* DSJ4 was isolated from the Ryukyu trench off the Japanese coast at 5 km and is capable of growth over a range of temperatures (4-18°C) and pressures (0.1-70 MPa) and grows optimally at 10°C

(10 MPa) (Nogi *et al.*, 1998b). *P. profundum* 3TCK is a pressure-sensitive strain isolated from a shallow water sediment sample in San Diego bay, USA (Campanaro *et al.*, 2005). *P. profundum* 3TCK is capable of growth over a range of temperatures (0->20°C) and grows optimally at atmospheric pressure. Little work has been performed on DSJ4 and 3TCK and as such it is difficult to draw comparisons. A genomic comparison demonstrated that while SS9, 3TCK and DSJ4 have significant similarities in genomic organisation which are generally shared throughout the *Vibrionaceae* (Heidelberg *et al.*, 2000), there are some significant differences. By comparing the open reading frames (ORFs) present in the SS9 genome to the DSJ4 and 3TCK genomes, 562 were absent in DSJ4 and 544 were absent in 3TCK. ORFs which were absent in DSJ4 and 3TCK include genes involved in fatty acid biosynthesis, lateral flagella, xylose transport, etc. (Campanaro *et al.*, 2005). In both DSJ4 and 3TCK, chromosome 2 had a higher proportion of the missing ORFs. This feature is true for other *Vibrionaceae*, where chromosome 1 contains established genes and chromosome 2 is the target for more gene transfer events, which is true for other *Vibrionaceae* (Heidelberg *et al.*, 2000). Interestingly, in the piezophilic strains SS9 and DSJ4 there were 171 ORFs that were absent or divergent in the piezosensitive strain 3TCK (Campanaro *et al.*, 2005). Of these ORFs, 6 were found to be upregulated at high pressures. Highlighting the importance of the membrane composition, one region absent in both 3TCK and DSJ4 contains *flm* genes, which in other bacteria are required for O-antigen biosynthesis and flagellar filament assembly. This region was found to be upregulated at 28 MPa and differentially expressed at 4°C.

The *P. profundum* SS9 genome was also shown to contain three regions which are typical of genomic islands, with GC content anomalies, altered codon bias and the presence of an integrase at one end (Campanaro *et al.*, 2005). Intriguingly, a large part of the genes located in these regions lack orthologous genes in other bacteria. This suggests that these genes have been acquired from still unknown bacteria. The second chromosome of *P. profundum* SS9 also contains a plasmid integration element. This element contains various bacterial conjugation factors and a multidrug efflux system.

1.4.5 Outer membrane fatty acid composition

As well as proteins, the composition of the outer membrane was also found to play an important role in the extremophilic growth of *P. profundum* SS9. The composition of the outer membrane was shown to alter with respect to temperature and pressure (Allen *et al.*, 1999; Allen and Bartlett, 2000). The unsaturated fatty acid C18:1 was found to increase and C16:0 and C16:1 were found to decrease with respect to high pressures. Like in other bacteria, the proportion of unsaturated fatty acids increased with a reduction in temperature and an increase in pressure. In *E. coli*, FabF (β -ketoacyl-ACP-synthase II) catalyses the elongation of C16:0 to C18:1. By disrupting *fabF* and by using cerulenin (inhibits formation of unsaturated fatty acids by inhibiting FabF), C18:1 was discovered to be required for piezophilic growth (Allen *et al.*, 1999; Allen and Bartlett, 2000).

1.4.6 Transposon mutant screen of *P. profundum* SS9

In order to investigate the mechanisms of high pressure and cold temperature adaptation in *P. profundum* SS9, random transposon mutagenesis was performed (Lauro *et al.*, 2008). Transposons are DNA sequences flanked by inverted repeat sequences. The transposon can encode for a transposase or one can be encoded near by. The transposase and the inverted repeats allow the transposon to excise and relocate itself within the genome. Transposons are useful for mutagenesis screens because of the degree of randomness with which the transposons integrate. In this case, mini-Tn5 and mini-Tn10 transposons were used, this was because such forms of transposons are generally accepted to be more stable and have been experimentally shown to have little or no downstream effects (Larsen *et al.*, 2002). The transposon mutants were then screened for a desired phenotype, such as a reduced growth rate at 4°C (0.1 MPa) or at 45 MPa (17°C) compared to the parent strain. From the mutants that were selected in the cold temperature or high pressure sensitive screens, the transposon was located within the genome by arbitrarily primed PCR and the disrupted gene was identified. From the screen, 31 mutants were isolated which contained transposon insertions in

genes involved in a variety of cellular mechanisms (Table 2). Genes identified from the cold sensitivity screen were involved in signal transduction, cell envelope biosynthesis, metabolism and protein export and synthesis. Genes identified from the pressure sensitivity screen were involved in chromosome replication, ribosomal biosynthesis and structure and 2 previously identified genes (FL4-*rpoE* and FL30-acyl carrier protein). From the cold temperature sensitive screen it could be seen that a number of mutants contained transposon insertions in genes which encode proteins putatively involved in polysaccharide biosynthesis. In this thesis, two of these mutants, FL26 and FL9, were further characterised.

Table 2: Transposon mutants isolated from random transposon mutagenesis

Mutant	Gene	Annotation	Type of transposon insertions recovered in ORF	Phenotype
FL1	PBPRB2014	Transcriptional regulator; LuxR family	a-Tn5, b-Tn10	CS
FL2	PBPRB0212	ATP-dependent DEAD box RNA helicase	Tn5	CS
FL3	PBPRA2678	Hypothetical protein	a-Tn5, b-Tn5	CS
FL4	PBPRA3093	<i>spoE</i> regulatory protein RseB	Tn5	CS, PS
FL5	PBPRA2681	Glycosyl transferase	a-Tn5, b-Tn5	CS
FL6	PBPRA1774	ATP-dependent protease; LA related	Tn10	CS
FL7	PBPRA2684	Hypothetical protein involved in polysaccharide biosynthesis	Tn5	CS
FL8	PBPRA0396	Hypothetical integral membrane protein	a-Tn5, b-Tn5	CS
FL9	PBPRA2686	Tyrosine protein kinase	Tn5	CS
FL10	PBPRA0428	Pyruvate kinase I	a-Tn10, b-Tn10	CS, PS
FL11	PBPRA1082	DNA-binding protein H-NS	Tn5	CS, PE
FL12	PBPRA0747	Suppressor protein SuhB	Tn10	CS, PS
FL13	PBPRA0189	Guanosine-3,5-bis(diphosphate) 3-pyrophosphohydrolase SpoT	Tn5	CS, PS
FL14	PBPRA2407	Periplasmic linker protein	Tn5	CS
FL15	PBPRA3239	Hypothetical protein	Tn5	CS
FL16	PBPRA2282	Conserved hypothetical protein	Tn5	CS
FL17	PBPRB2009	Phosphotransferase cellobiose-specific component IIC	Tn10	CS
FL18	PBPRA0667	Conserved hypothetical protein	Tn10	CS
FL19	PBPRB1941	Hypothetical protein	Tn10	CS
FL20	Unknown	Transposase	Tn10	CS
FL21	PBPRB1757	Hypothetical protein	Tn10	CS
FL22	PBPRB0828	Beta-hexosaminidase	Tn10	CS
FL23	PBPRA3229	Phosphoheptose isomerase	Tn10	CS, PS
FL24	PBPRA0917	Polar flagellar protein FlaJ	Tn10	CS
FL25	PBPRA2700	Hypothetical protein	a-Tn5, b-Tn5	CS
FL26	PBPRA0218	O-antigen ligase	Tn5	CS
FL27	PBPRA0674	Conserved hypothetical protein	Tn5	CS
FL28	PBPRA1039	SeqA protein	Tn10	PE
FL29	PBPRA2596	L-Asparaginase	a-Tn5, b-Tn5	PS
FL30	PBPRA2658	3-Oxoacyl-(acyl carrier protein) synthase I	Tn5	PS
FL31	PBPRB0001	Chromosome II replication protein RctB	Tn5	PS

CS, cold sensitive; PS, pressure sensitive; PE, pressure enhanced. [Table taken from (Lauro *et al.*, 2008)]

1.4.7 The *P. profundum* SS9 putative O-antigen ligase mutant, FL26

As previously mentioned, random transposon mutagenesis was performed to identify genes in *P. profundum* SS9 that were involved in high pressure and cold temperature adaptation (Lauro *et al.*, 2008). Of the mutants identified in the cold temperature sensitive screen, FL26 was found to contain a mini-Tn5 transposon insertion in *pbpra0218*, a gene which encodes a protein with significant homology to O-antigen ligase. O-antigen ligase is required for the addition of the O-antigen polymer onto the lipid A-core (Abeyrathne *et al.*, 2005; Heinrichs *et al.*, 1998a; Klena *et al.*, 1992; Schild *et al.*, 2005). Mutants lacking O-antigen ligase have been shown to have an altered LPS profile by SDS-PAGE (Abeyrathne *et al.*, 2005; Heinrichs *et al.*, 1998b; Schild *et al.*, 2005). In *Salmonella* and *E. coli* species this is a loss of the heterogeneous ladder pattern, resulting in a single band. In *P. aeruginosa*, O-antigen ligase mutants are also non-motile due to a loss of pili and flagella (Abeyrathne *et al.*, 2005), although this defect is not common within other bacterial species. For example, *Salmonella* O-antigen ligase mutants have lost swarming motility, but maintain swimming motility (Toguchi *et al.*, 2000).

The composition of the outer membrane is of great importance to bacteria. The outer membrane prevents the diffusion of hydrophobic molecules, such as detergents (SDS and DOC), antibiotics (rifampicin and actinomycin) and dyes (eosine and methylene blue) (Nikaido and Vaara, 1985). By changing the composition of the outer membrane, the sensitivity of bacteria to certain stresses can therefore be increased. Changes in the phospholipid content have been shown to affect the temperature dependence of certain membrane enzymes (Mavis and Vagelos, 1972). By the addition of various unsaturated fatty acids, the activity of an *E. coli* acetyltransferase at certain temperatures could be modulated. As well as enzyme activity, alterations in LPS composition (such as *Salmonella* deep rough mutants, mutants which have an alteration in the inner core of LPS) can also affect the overall protein composition of the outer membrane (Ames *et al.*, 1974).

1.4.8 LPS transport and the role of O-antigen ligase

The LPS core precursor is synthesised on the cytoplasmic face of the inner membrane (Mulford and Osborn, 1983) where it is then translocated across the inner membrane (McGrath and Osborn, 1991a) by MsbA (Doerrler *et al.*, 2004; Zhou *et al.*, 1998). O-antigen is attached to the membrane-bound carrier, undecaprenyl diphosphate (und-PP), at this point it is separate to the lipid A-core. O-antigen is then transported across the inner membrane by one of three mechanisms: Wzy-, synthase- or ABC transporter-dependant pathways (Raetz and Whitfield, 2002). Since BLAST analyses have shown that the *P. profundum* SS9 genome does not encode a protein with any significant homology to Wzy, this method will not be discussed. Synthases are capable of synthesising entire polymers within a single polypeptide chain. Included in the synthase family are the enzymes involved in cellulose and chitin biosynthesis and the hyaluroic acid capsule from *Streptococcus pyogenes*. However, there is only one example of a synthase-dependant O-antigen pathway, which is found plasmid-encoded in *Salmonella enterica* serovar Borreze (Keenleyside and Whitfield, 1996). This leaves ABC transporter-dependant pathways, which involve progressive addition of glycosyl residues to the growing und-PP linked O-antigen chain (Guan *et al.*, 2001) (Figure 7). To date, all O-antigen polysaccharides formed by this pathway have been initiated by WecA. WecA synthesises an und-PP linked GlcNAc which acts as a primer to O-antigen synthesis (Rick *et al.*, 1994; Susskind *et al.*, 1998). ABC transporter-dependant systems have previously been identified for the export of LPS in *Vibrio cholerae* (Manning *et al.*, 1995). This combined with the *P. profundum* SS9 genome containing proteins with significant homology to WecA (*pbpra2673* is 70% similar to *Klebsiella pneumoniae* 342 WecA) makes it likely that *P. profundum* SS9 uses the ABC transporter-dependant pathway. Once the O-antigen polymer is completed at the cytoplasmic face of the inner membrane it is exported to the periplasmic face by an ABC transporter, typically formed by the proteins Wzm and Wzt. O-antigen ligase accepts the O-antigen polymer and ligates it to the lipid A-core molecule, which occurs on the periplasmic face of the inner membrane (Mulford and Osborn, 1983) by a still incompletely understood mechanism (Kaniuk *et al.*, 2004).

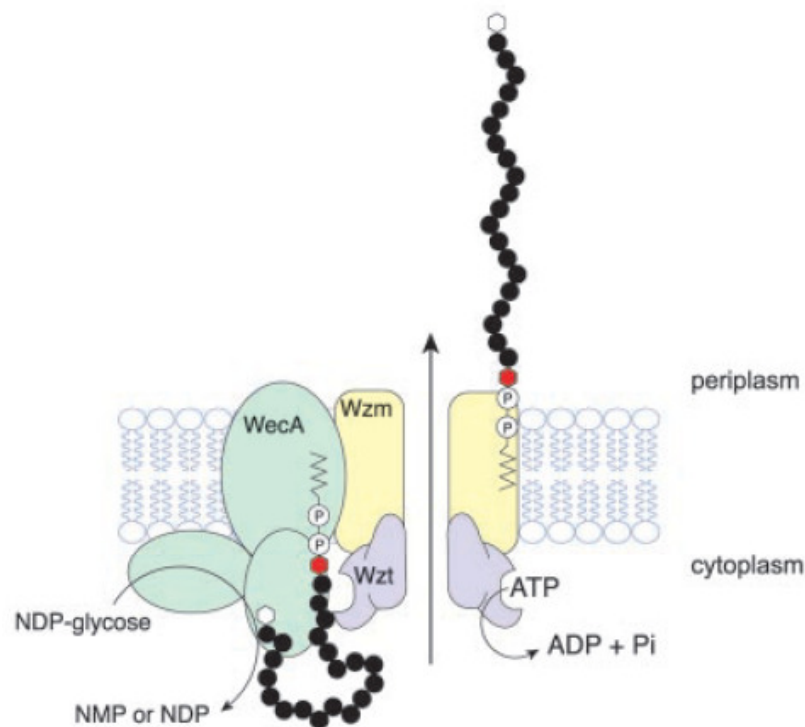


Figure 7: ABC transporter dependant mechanism of LPS assembly and transport in *E. coli*

WecA synthesises Und-PP-GlcNAc which acts as a primer for glycosyl addition, shown in red. Glycosyltransferases are shown in green, ABC transporter formed by Wzm (yellow) and Wzt (blue) are required for transport of undecaprenyl-linked polymer to the periplasmic face of the inner membrane. From here it is ligated to the lipid A-core and transported to the outer membrane. [Figure from (Raetz and Whitfield, 2002)].

The completed LPS molecules are then transported across the periplasm to the outer membrane by a still unknown mechanism (Doerrler, 2006) (Figure 8). Several proteins, have been suggested to be involved in LPS transport to the outer membrane including LptA and B and MsbA. LptA and B have been shown to be required for LPS transport to the outer membrane, the LPS is instead localised in a novel membrane fraction which has a density between the inner and outer membranes (Sperandeo *et al.*, 2007). MsbA is an essential ABC transporter in *E. coli* and has been shown to be required for the translocation of phospholipids and Lipid A from the cytoplasmic to the

periplasmic face of the inner membrane (Doerrler *et al.*, 2001; Doerrler *et al.*, 2004; Doerrler, 2006). Also, some positive evidence suggests completed LPS molecules are transported via spheroplasts from the IM to OM with a requirement for MsbA (Tefsen *et al.*, 2005). Finally, the LPS molecules are transported across the outer membrane, this has been suggested to involve Imp. Imp is an essential outer membrane protein in *E. coli* and its depletion results in abnormalities in the outer membrane (Martin and Thomas, 2002). An Imp mutant in *Neisseria meningitidis* produced reduced amounts of LPS, but more importantly this LPS was inaccessible to LPS modifying enzymes in the outer membrane which demonstrated that Imp plays a role in the transport of LPS to the outer membrane (Bos *et al.*, 2004). Imp has also been shown to interact physically with RlpB, depletion of which results in outer membrane abnormalities similar to those seen with Imp (Wu *et al.*, 2006).

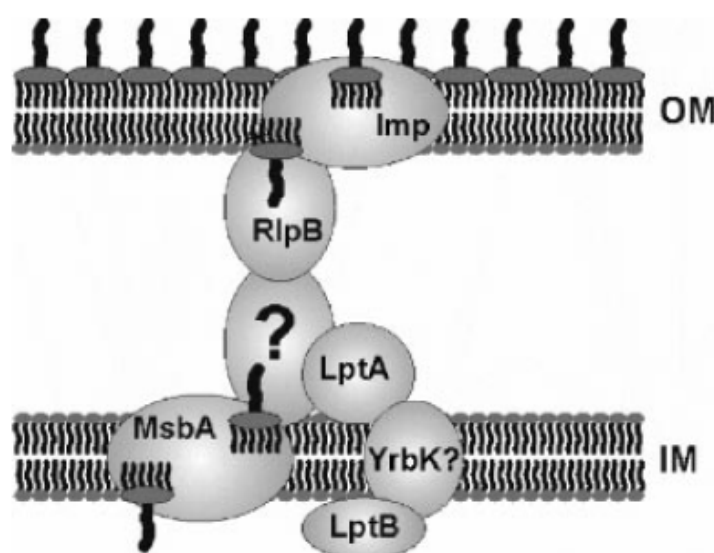


Figure 8: Postulated LPS transport to the outer membrane in gram negative bacteria

Lipid A-core is synthesised on the cytoplasmic face of the inner membrane and transported across to the periplasmic face by MsbA. Here, O-antigen is added and the completed LPS molecule is transported across the periplasm by a still unknown mechanism. The LPS is then transported to the periplasmic face of the outer membrane by LptA and B, possibly involving a transmembrane partner such as YrbK. Finally the LPS is translocated across to the outer face of the outer membrane, possibly by Imp and RlpB. [Figure adapted from (Sperandeo *et al.*, 2007)].

1.4.9 The *P. profundum* SS9 putative tyrosine protein kinase mutant, FL9

From the previously mentioned random transposon mutant screen of *P. profundum* SS9, the cold sensitive mutant FL9 was isolated (Lauro *et al.*, 2008). FL9 was found to contain a mini-Tn5 transposon insertion in *pbpra2686*, a gene which encodes a protein with significant homology to the tyrosine protein kinase, Wzc. Wzc is required for the polymerisation and transport of capsular polysaccharide (CPS) to the cell surface (Drummelsmith and Whitfield, 1999) and mutations in *wzc* therefore lead to a loss of CPS expression (Wugeditsch *et al.*, 2001). While a loss of CPS has not previously been demonstrated to affect cold temperature sensitivity, EPS has been shown to be important for survival of desiccation and reducing freeze-thaw damage (Gibson *et al.*, 2006; Ophir and Gutnick, 1994; Tamaru *et al.*, 2005). In group 1 *E. coli*, mutations in *wzc* can also affect the LPS composition. In the absence of Wzc the CPS subunits are still synthesised to a small degree and they can be added to the LPS to form KLPS (MacLachlan *et al.*, 1993). A similar alteration has also been demonstrated in *Vibrio cholerae* 0139, in which capsular repeat units were detected as part of the LPS (Cox and Perry, 1996).

1.4.10 The role of Wzc in CPS transport

In *E. coli*, Wzc is a tyrosine protein kinase that is required for the polymerisation and expression of capsular polysaccharide (CPS) (Drummelsmith and Whitfield, 1999). At least four different types of capsule are known in *E. coli*, two of which utilise Wzc in the expression of CPS, group 1 and 4. Group 1 and group 4 capsules are formed by the protein complex of Wza, Wzb, Wzc, Wzx, Wzy and Wzi (Drummelsmith and Whitfield, 1999; Peleg *et al.*, 2005). Group 1 requires the addition of glycosyl residues to und-PP by WbaP (Wang *et al.*, 1996). Group 4 requires a similar process which is performed by WecA (Price and Momany, 2005). Und-PP linked oligomers are flipped across the inner

membrane by Wzx, to the periplasmic face. Wzy-dependant polymerisation acts by the transfer of the growing polysaccharide chain onto the incoming und-PP linked oligomer. Polymerisation requires the cyclic autophosphorylation of Wzc and its dephosphorylation by Wzb (Vincent *et al.*, 2000). Wzc forms a channel with Wza across the periplasm to the outer membrane, through which completed CPS is transported (Dong *et al.*, 2006; Reid and Whitfield, 2005). The CPS is then anchored without any currently known lipid linker to the outer membrane. Wzi has been shown to influence the association of CPS with the outer membrane (Rahn *et al.*, 2003).

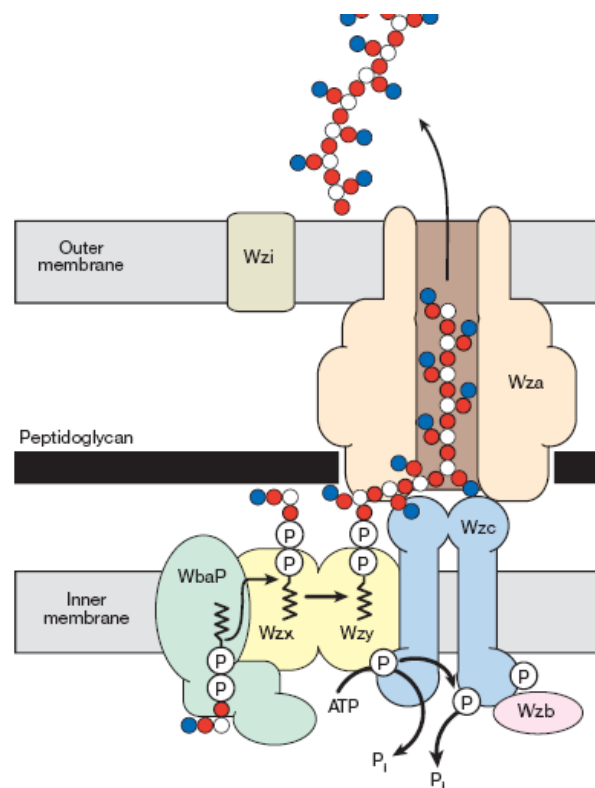


Figure 9: Assembly of capsular polysaccharides in *E. coli* group 1 and 4 capsules

Wzx flips CPS subunits across inner membrane. Wzy and Wzc interact to polymerise CPS. Kinase and phosphatase activity of Wzc Wzb, respectfully, also act to influence the polymerisation of CPS. Wzc and Wza form a channel through which CPS are transported to the cell surface. Wzi is unique to group 1 capsules and is known to influence the association of CPS with the cell surface. Red, white and blue circles represent capsular subunits. [Figure from (Whitfield, 2006)].

1.5 Aims and objectives

The overall aim of this project was to understand more about the high pressure and cold-adapted growth of *P. profundum* SS9. The main hypothesis was that surface polysaccharides play an important role in the cold-adapted growth of *P. profundum* SS9.

The specific objectives of the project were:

- 1 To develop methods for the growth of *P. profundum* SS9 in liquid and on solid media, at both high and low pressures
- 2 To characterise the surface polysaccharides of *P. profundum* SS9 from growth under conditions of high and low pressure and cold temperatures
- 3 To further characterise the *P. profundum* SS9 putative polysaccharide biosynthesis mutants, FL26 and FL9

2. Materials and Methods

2.1 Bacterial plasmids and strains

Bacterial plasmids and strains used in this study are described in Table 3 and Table 4, respectively.

Table 3: Plasmids used in this study

Plasmid	Description	Reference
pREP4	LacI repressor, Kan ^R ,	(QIAGEN)
pQE30	T7 IPTG inducible expression vector, 6x His tag, Amp ^R	(QIAGEN)
pETDEST42	T7 IPTG inducible expression vector, 6x His tag, Amp ^R	(Invitrogen)
pRK2013	Carries <i>tra</i> genes for conjugal transfer, Kan ^R	(Figurski and Helinski, 1979)
pFL202	pQE30Xa + <i>pbpra3129</i> -His	D. Bartlett (Scripps, San Diego, USA)
pDA05	pQE30 + <i>pbpra1210</i> -His	F. Lauro (Scripps, San Diego, USA)
pFL190	Broad host range arabinose-inducible vector, Strep ^R	This study (Lauro <i>et al.</i> , 2005)
pFL191	pFL190 + LacZ	(Lauro <i>et al.</i> , 2005)
pDA01	pFL190 + <i>pbpra0218</i>	This study
pDA02	pFL190 + <i>pbpra2686</i>	This study
pWQ310	pBAD24 containing O-antigen ligase gene from SARC 1	(Kaniuk <i>et al.</i> , 2004)
pDA03	pFL190 + O-antigen ligase gene from SARC 1	This study
pDA04	pFL190 + <i>pbpra0217-0218</i>	This study

Table 4: Bacterial strains used in this study

Strain	Description	Reference
<i>Photobacterium profundum</i> SS9	A deep sea isolate, piezophilic and psychrophilic	(DeLong, 1986)
SS9R	Rifampicin resistant variant of SS9, Rif ^R	(Chi and Bartlett, 1993)
FL3	SS9R <i>pbpra2678::Tn5</i> (encodes putative glycosyltransferase), Kan ^R	(Lauro <i>et al.</i> , 2008)
FL5	SS9R <i>pbpra2681::Tn5</i> (encodes putative glycosyltransferase), Kan ^R	“
FL7	SS9R <i>pbpra2684::Tn5</i> (encodes putative “Flipase”, Wzx), Kan ^R	“
FL9	SS9R <i>pbpra2686::Tn5</i> (encodes putative tyrosine protein kinase, Wzc), Kan ^R	“
FL25	SS9R <i>pbpra2700::Tn5</i> (encodes putative CDP glycerolphosphate glycerotransferase, “TagF”), Kan ^R	“
FL26	SS9R <i>pbpra0218::Tn5</i> (encodes putative O-antigen ligase, WaaL), Kan ^R	“
3TCK	Pressure-sensitive psychrophilic <i>P. profundum</i> strain	(Campanaro <i>et al.</i> , 2005)
<i>Escherichia coli</i> M15	<i>lacZ</i> ⁻	(Marinkovic and Tang, 1971)
DH5α	<i>supE44 ΔlacU169 (Φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	(Hanahan, 1983) Molecular Cloning 2 nd Ed
BL21 DE3 CSH56	F ⁻ <i>ompT hsdS_B (r_B⁻m_B⁻) gal dcm rne131 araΔ(lacpro) supD nalA thi</i>	(Invitrogen) D. Bartlett (Scripps, San Diego, USA) Experiments in Molecular Genetics
CWG258	E69 (O9a:K30) with <i>wza</i> _{22 min} :: <i>aadA</i> insertion (polar on <i>wzb</i> _{cps} and <i>etk</i>); Sp ^r	(Drummelsmith and Whitfield, 2000)
CWG285	E69 with <i>wzc</i> _{cps} :: <i>aacC1</i> and <i>wza</i> _{22 min} :: <i>aadA</i> insertion (polar on <i>wzb</i> _{cps} and <i>etk</i>); Gmr, Sp ^r	(Wugeditsch <i>et al.</i> , 2001)
<i>Salmonella enterica</i> serovar Typhimurium G95 LT2 SL3749	Wild type isolate S. Typhimurium LT2 <i>waaL446</i> ; R-LPS	(Kaniuk <i>et al.</i> , 2004) (Kaniuk <i>et al.</i> , 2004) SGSC228

2.2 Antibiotics

Kanamycin (sigma, K400), rifampicin (R3501), ampicillin (Sigma A9518) and streptomycin were suspended in distilled water and filter sterilised using a 0.2 µm pore syringe filter. Stock solutions concentrations are described in Table 5.

Table 5: Antibiotic stock solutions

Antibiotic	Stock (mg.ml ⁻¹)	Final concentration (µg.ml ⁻¹)	
		<i>E. coli</i> and <i>Salmonellae</i>	<i>P. profundum</i> SS9
Kanamycin	25	50	200
Rifampicin	50	100	
Ampicillin	100	100	
Streptomycin	500	100	150 / 100

2.3 Media

2.3.1 Luria-Bertani (LB) broth and Agar

To make 1 litre, 10 g tryptone, 5 g yeast extract, 10 g of NaCl, and for agar, 15 g of Bacto™ agar (Becton Dickinson, 214010). LB agar plates were allowed to set and then dried in a 65°C incubator before use.

Motility LB agar was prepared exactly above, with the exception that 0.3% (w/v) Bacto™ agar was added to the LB base. LB motility plates required several hours to set, plates were therefore left overnight at room temperature and used the following day. Motility plates were not dried in 65°C incubator as with all other plates.

2.3.2 Marine broth and Agar

Marine broth was made as previously described (Chi and Bartlett, 1993) with a few minor changes. 28 g Marine broth (Becton and Dickinson, Difco 2216) was added to 800 ml distilled water and boiled for 5 minutes. After cooling, the broth was filtered using grade 1, 11 µm pore qualitative cellulose filters (Whatman). The media was buffered using 100 mM HEPES (Sigma, H4034) and adjusted to pH 7.5 using

10 M NaOH. The volume was made up to 1 litre and then autoclaved. On cooling, 20 mM glucose was added prior to use.

To make marine agar (and any derivation thereof) the marine broth and agar were made double strength and autoclaved separately, i.e. 28 g marine broth in 500 ml distilled water buffered with 200 mM HEPES (pH 7.5) and an agar solution containing 17 g Bacto™ agar in 500 ml distilled water. These solutions were mixed after autoclaving to give marine agar. On cooling, 20 mM glucose was added before use and 25 ml of the final solution was poured for each plate. Marine agar plates were allowed to set and then dried in a 65°C incubator.

Motility marine agar was prepared exactly as above, with the exception that the agar solution was 0.6% (w/v), to give a final concentration of 0.3% (w/v). Motility plates required several hours to set, plates were therefore left overnight and used the following day. Marine motility plates were not dried in 65°C incubator as with all other plates.

For β -galactosidase activity, 100 $\mu\text{g.ml}^{-1}$ streptomycin, 40 $\mu\text{g.ml}^{-1}$ X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) and 0.01 – 1.0% (w/v) arabinose were added as required.

2.3.3 Low temperature gelling marine agar

Low Temperature Gelling Agar (LTGA) (Nacalai tesque, 01059-85) was required for overlay plates. Low Temperature Gelling Marine Agar (LTGMA) was made by the same procedure described for marine agar, however the agar was substituted for LTGA at 5% (w/v) [2.5% (w/v) final]. On cooling, 20 mM glucose was added to LTGMA before use and 25 ml of the final solution was poured for each. A portion of LTGMA was kept molten at 50°C. LTGMA plates were allowed to set and then dried in a 65°C incubator. *P. profundum* SS9 cultures were spotted onto the plates as described in results and once dry were incubated at 15°C (0.1 MPa) for 2 hours. The plates were then overlaid with the molten LTGMA which set almost immediately. The overlaid plates were briefly stored at 15°C until they were removed from the petri dish

and placed in a sterile plastic pouch (Vacuum Pouch Co.). The pouch was heat sealed (Hualian FS-300) and incubated under the defined conditions.

2.4 Culture conditions

2.4.1 *Escherichia coli*

All *E. coli* strains were cultured in Luria-Bertani (LB) broth with the appropriate antibiotics. Strains containing pREP4 were grown in the presence of 50 $\mu\text{g}\cdot\text{ml}^{-1}$ kanamycin, and strains with either pQE30 or pETDEST42 were grown with 100 $\mu\text{g}\cdot\text{ml}^{-1}$ ampicillin. Relevant growth temperatures are given in the Results section.

2.4.2 *Photobacterium profundum* SS9 and 3TCK

Marine broth was pre-chilled to 15°C before use. 20 μl of frozen stock culture was added to 5 ml marine broth. Liquid cultures were prepared either aerobically in shaking test tubes or anaerobically in heat sealed plastic Pasteur pipettes. Anaerobic cultures were syringed into sterile plastic Pasteur pipettes (Fisher, FB55349) and heat sealed (Hualian FS-300), making sure to remove any excess air bubbles from the culture.

P. profundum cultures were grown at 15°C and either 28 or 0.1 MPa. These cultures were incubated for 48 hours before being removed onto ice and diluted to an OD₆₀₀ of 0.1 in fresh marine broth. The diluted cultures were once more transferred into bulbs, sealed and incubated under the desired conditions. When large volumes were required, a sterile plastic bag (Vacuum Pouch Co.) was used to hold up to 1 litre of culture. For growth curves, a plastic bag was inoculated with 200 ml marine broth and *P. profundum* at OD₆₀₀ 0.1. Periodically a needle was used to puncture the bag and remove a sample of culture for OD₆₀₀ measurement. The bag was resealed using a bag sealer.

To analyse growth on solid agar, the required *P. profundum* strain was pre-grown under defined conditions, diluted to OD₆₀₀ of 0.2, serially diluted and spotted in

triplicate onto marine agar plates with glucose. Once the culture spots had dried the plates were inverted, wrapped in foil and incubated under the desired conditions.

To analyse growth on agar at pressure, the required *P. profundum* strain was pre-grown under defined conditions, diluted to OD₆₀₀ of 0.8 and serially diluted. These dilutions were spotted in triplicate onto a 2.5% (w/v) LTGMA plate. After allowing the spots to dry, the plate was incubated at 15°C for 2 hours. 2.5% (w/v) LTGMA was cooled to 50°C and 15 ml was poured over the LTGMA plate. Once set, the agar was then removed from the petri dish and transferred into a sterile plastic bag and sealed. The plate could then be incubated under the desired conditions.

The pressure vessel has a 5 litre capacity and distilled water was used to create the pressure. The vessel temperature was maintained by a circulating water jacket connected to a refrigerated water bath (Fisher Isotemp 1016S) (Figure 10). Pressurisation was achieved in ≤ 5 minutes and depressurisation in ≤ 2 minutes.

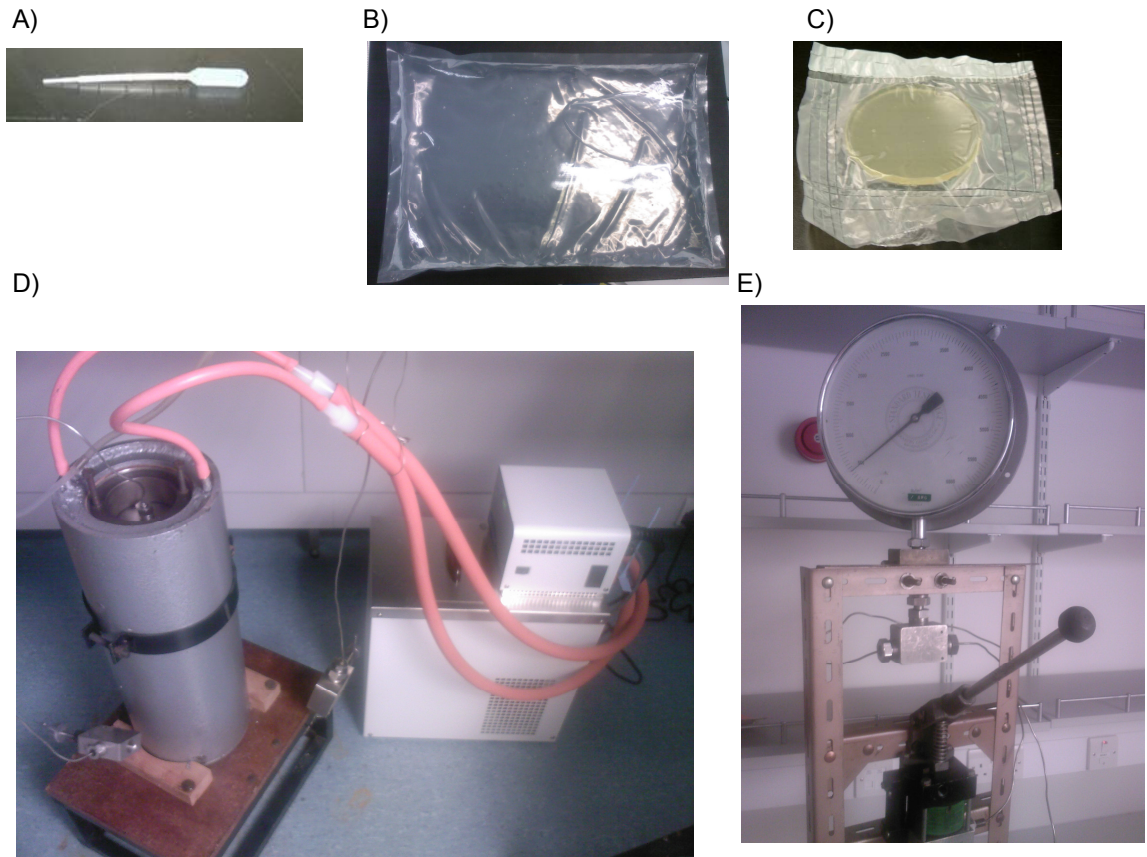


Figure 10: Incubating cultures at high pressures

Defined cultures were heat sealed in sterile plastic Pasteur pipettes (5 ml) (A) or plastic pouches (up to 1 litre) (B). For assays on solid agar, defined cultures were spotted onto low temperature gelling marine agar (LTGMA), overlaid with molten LTGMA and sealed in a plastic pouch (C) (method 2.3.3). These were then incubated at the desired temperature using a circulating water bath (D). Pressure was achieved via a manual pump (E).

2.5 Determination of colony forming units

A 100 μ l sample was taken from liquid cultures and serially diluted (10^{-1} - 10^{-6}) in 1 ml of fresh marine broth. 10 μ l of each dilution was pipetted in triplicate onto marine agar and incubated at 15°C. Colony forming units per ml (cfu.ml⁻¹) = No. of colonies \times dilution factor \times volume factor).

2.6 Motility Assay

Stationary phase cultures were diluted to OD₆₀₀ 1.0. 3 μ l of this suspension was pipetted onto the centre of a motility agar plate. The motility was assessed by frequent measurement of the longest and shortest diameters in order to average the movement. Typically, 3 plates were used for each culture assayed.

2.7 Basic Biofilm Assay

Stationary phase cultures were diluted to an OD₆₀₀ of 0.1 and 5 ml was pipetted into glass test tubes (Fisher). These were incubated at 15°C 0.1 MPa, 200 rpm. Every 24 hours the tubes were visually assessed for attachment of bacterial cells to the glass.

2.8 Microscopy

2.8.1 Light Microscopy

All images have been taken at 100x oil immersion phase contrast with a Carl Zeiss microscope and AxioCam using AxioVision version 2.0. Slides were pre-coated with poly-L-lysine (Sigma) to immobilise the bacteria, as per manufacturers guidelines. Briefly, the poly-L-lysine solution was diluted 1:20 in distilled water. Fresh glass slides were immersed in the solution for 5 minutes and allowed to air dry overnight. For a quicker coating, 500 μ l of the solution was pipetted onto the centre of a fresh slide, this was then dried for 1 hour in a 65°C incubator.

2.8.2 Lipid staining

A culture was spread onto a clean glass slide and heat fixed by passing through a blue bunsen flame. The cells were then covered with sudan black B solution (0.3% [w/v] in 70% [v/v] ethanol). This was allowed to air dry until all the ethanol in the solution had evaporated. The slide was then immersed in 100% (v/v) xylene until completely decolourised. This was then allowed to dry before being flooded with Fuchsin (0.5% [w/v] in distilled water), left for 10 seconds, then rinsed in distilled water and examined by light microscopy.

2.8.3 Flagella staining

The flagella were visualised using a published method (Clark, 1976). Glass slides needed to be thoroughly cleaned before use. They were shaken in an acidified ethanol mixture (3% [v/v] concentrated HCl in 100% [v/v] ethanol) for at least 4 days. The slides were thoroughly cleaned with distilled water and air dried. Dry slides were then passed through a blue Bunsen flame 5 times.

For every 5 ml bacterial sample, 250 µl 37% (v/v) formaldehyde was added. This was pelleted and the pellet was resuspended in 5 ml distilled water. The sample was pelleted again and resuspended in 2 ml distilled water. 500 µl of the prepared sample was then pipetted onto the clean microscope slides, and allowed to run along the length of the slide and to air dry. 1 ml fuchsin stain (1 volume of 1.2% [w/v] fuchsin in ethanol, with 2 volumes 0.75% [w/v] NaCl and 1.5% [w/v] tannic acid, pH of final solution was adjusted to 5.0) was pipetted onto the slide and left for 5-15 minutes. Excess stain was removed with distilled water and the slide was allowed to dry. The flagella were then examined by light microscopy.

2.8.4 Transmission Electron Microscopy

1 ml of liquid culture was pelleted in a bench top centrifuge (Fisher accuSpin MicroR) for approximately 30 seconds and the pellet was resuspended in 100 µl of 100 mM HEPES, pH 7.4. Cells were treated following a published method (Rahn *et al.*, 2003). This involved the suspension of the cells in 20 µl cationised ferritin (Sigma, F7879) (cationised ferritin resuspended in 0.5 ml 100mM HEPES buffer) to stabilise any capsule present. The samples were washed twice in 100 mM HEPES pH 7.5 and processed. Cells were initially fixed in modified Karnovsky fixative (Karnovsky, 1965) (2.5% [w/v] gluteraldehyde, 0.02% [v/v] 100mM CaCl₂, 2% [v/v] paraformaldehyde in 0.08 M Sodium cacodylate) for 1 hour. After washing in HEPES buffer, the material was post fixed in 2% (w/v) osmium tetroxide for 1 hour. Material was dehydrated in an alcohol series and infiltrated with spurrs resin. All samples, when polymerised, were cut using a Leica UCT ultramicrotome to produce 90 nm thickness (gold) sections. These were collected on 300 mesh copper grids and stained using aqueous uranyl acetate then counterstained with Lead citrate. Imaging of the sections was undertaken on a Philips (FEI) CM 120 Biotwin TEM at 100 kV. Images were taken on Kodak SO163 electron microscope film.

2.9 LPS extraction and analysis

2.9.1 LPS extraction by SDS lysis

In order to isolate lipopolysaccharide (LPS) from *P. profundum* cultures, colonies were taken from solid agar cultures or 1 ml liquid culture in a defined growth phase was pelleted by centrifugation ((Fisher accuSpin MicroR) and resuspended in 30 µl lysis buffer (1 M tris-Cl, pH 6.8, 2% [w/v] SDS, 4% [v/v] β-mercaptoethanol, 10% [v/v] glycerol, 0.005% [w/v] bromophenol blue). The sample was then boiled for 10 minutes. After cooling to room temperature, 10 µl proteinase K (Sigma P-2308, 2.5 mg.ml⁻¹ stock) was added and the sample heated at 60°C (1 hour). 80 µl of sample buffer (120 mM tris-HCl pH 6.8, 3% [w/v] SDS, 9% [v/v] β-mercaptoethanol, 30% [v/v]

Glycerol, 0.03% [w/v] bromophenol blue) was then added. Samples were stored at -20°C until analysed by SDS-PAGE. For long term storage samples were kept at -80°C.

2.9.2 LPS extraction by hot phenol water

Using a modified method (Westphal and Jann, 1965), a 5 ml culture was grown to stationary phase and 4 ml of this was used to inoculate a 1 litre culture. The culture was grown to stationary phase ($OD_{600} \sim 1$ in *P. profundum* after 48 hours). The cells were harvested by centrifugation at 9000 g for 20 minutes (DuPoint Sorval RC-5 and GSA rotor). Pellet was washed twice in 0.9% (w/v) NaCl. The pellet was resuspended in 70 ml chilled EDTA solution (0.05 M $Na_2HPO_4 \cdot 7H_2O$, 0.005 M EDTA, pH adjusted to 7). The mixture was sonicated on ice for 30 seconds at 80% with a 1 minute pause, repeating 10 times (Misonix 3000). Then 15 mg lysozyme (Fluka, 62971) was added and stirred in the cold room over night. The mixture was then heated to 37°C for 20 minutes with frequent stirring. 0.5 mg DNase (Sigma D5025), 10 mg RNase (R5503) and 25 ml of 0.04 M $MgCl_2 \cdot 6H_2O$ was added. The solution was then incubated at 37°C for 30 minutes followed by incubation at 60°C for 10 minutes. The mixture and 100 ml 80% w/w phenol were both heated to 65°C; they were then added together in a fume hood and heated at 65°C for 15 minutes with stirring. The mixture was then incubated on ice for 15 minutes and then centrifuged at 9000 g for 20 minutes in new polypropylene centrifuge tubes. The water and phenol layers were each pipetted into a new centrifuge tube. 30 cm lengths of dialysis tubing (Spectrapor 132655) were rinsed in distilled water and then filled with each of the layers and dialysed against 5 litres distilled water. The distilled water was changed 3 times a day, for 5 days. The dialysed samples were then centrifuged again at 9000 g and frozen at -80°C before being freeze-dried (Edwards, modulyo freeze dryer).

The initial freeze-dried raw LPS sample was resuspended in distilled water to a final concentration of about 5 mg.ml⁻¹ (the maximum concentration for this procedure is 10 mg.ml⁻¹). If the LPS did not resuspended properly, it was supersonified for 5 min in an ultrasonic bath. The suspension was spun at 5000 x g for 15 minutes prior to

ultracentrifugation to remove debris. The pellet contained majorly proteins and unwanted material. The supernatant was transferred to ultracentrifugation (Beckman Coulter, 331372) tubes and balanced to within 0.05g. These were ultracentrifuged for 3h 30min at 100,000 x g at 10°C (Beckman Coulter, Optima™ L-80XP Ultracentrifuge, SW41Ti Rotor). The supernatant was kept for freeze-drying, since it would majorly contain EPS and other glucans. The gelatinous pellet was resuspended in 1 ml distilled water and left to resuspend overnight in the fridge. All samples were then freeze dried and stored at room temperature until analysed by DOC-PAGE.

2.9.3 LPS extraction by hot phenol water, small scale

Using a modified method (Westphal and Jann, 1965), a 5 ml culture was grown to stationary phase, the OD₆₀₀ was recorded and the cells were harvested by centrifugation (Fisher accuSpin MicroR). The cells were resuspended in 0.5 ml distilled water and 0.5 ml phenol was added. The mixture was incubated at 60°C for 15 minutes, and vortexed every 5 minutes. The mixture was then incubated on ice for 15 minutes. The water and phenol layers were each pipetted into clean 5 cm lengths of dialysis tubing (Spectrapor 132655) and dialysed against 1 litres distilled water. The distilled water was changed 3 times a day, for 5 days. The dialysed samples were then frozen at -80°C before being freeze-dried (Edwards, modulyo freeze dryer).

2.9.4 LPS analysis by SDS-PAGE

The constituents of the gel used in Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) are described below (Table 6).

Table 6: Buffers required to make 2 mini gels for SDS-PAGE of LPS

Ingredients	Concentrating gel 15.5% (ml)	Separating gel 2.3% (ml)
distilled water	4.2	2.3
Gel buffer pH 8.45 (3 M tris, 0.3% [w/v] SDS)	1.5	3.32
Glycerol		1.04
46.5% (w/v) acrylamide, 3% (w/v) bis acrylamide sol ⁿ	0.5	3.32
10% (w/v) APS	0.130 (130 μ l)	0.040 (40 μ l)
TEMED	0.0073 (7.3 μ l)	0.004 (4 μ l)

The volume of sample loaded was varied according to optical density of the original sample. The gel was run using 0.2 M tris-HCl, pH 8.9, as the anode buffer and 0.1 M tris, 0.1 M tricine, 0.1% (w/v) SDS as the cathode buffer at 100 V for 3.5 hours (Mini-Protean[®] 3 and Bio-Rad PowerPac[™] Basic). The gel was removed and visualised by silver staining.

2.9.5 LPS analysis by DOC-PAGE

Samples were resuspended in sample buffer (4ml buffer C (Table 7), 5 mg bromophenol blue, 2 ml glycerol, made to 20 ml with distilled water). The constituents of the gel used in deoxycholate polyacrylamide gel electrophoresis (DOC-PAGE) are described in Table 7 below. The apparatus was filled with running buffer (21.7 g.l⁻¹ glycine, 4.5 g.l⁻¹ tris base, 2.5 g.l⁻¹ deoxycholic acid) and pre-ran at 30 mA for 10 minutes before loading the samples. Once loaded, the gel was ran at 30 mA for 1 hour (Mini-Protean[®] 3 and Bio-Rad PowerPac[™] Basic). The gel was removed and visualised by silver staining.

Table 7: Buffers required to make 2 mini gels for DOC-PAGE of LPS

Ingredients	Concentrating gel 18% (ml)	Separating gel 4% (ml)
H ₂ O	1.67	2
30% (w/v) acrylamide, 0.8% (w/v) bis acrylamide sol ⁿ	0.33	6
Buffer B (187 mM tris, pH 8.8)		2
Buffer C (63.5 mM tris, pH 6.8)	0.5	
10% (w/v) APS	0.0125 (12.5 µl)	0.0175 (17.5 µl)
TEMED	0.00625 (6.25 µl)	0.00875 (8.75 µl)

2.9.6 Visualising SDS-PAGE by silver staining

For sodium-meta-periodate silver staining, the LPS gels were incubated overnight in fixative solution (40% [v/v] ethanol, 5% [v/v] acetic acid). To visualise sugar residues, the gel was covered with 50 ml of oxidiser (0.7% [w/v] sodium-meta-periodate in fixative). After 5 minutes, the gel was washed 3 × 15 minutes in distilled water. The gels were silver stained (140 ml distilled water, 280 µl 10 M NaOH, 2 ml ammonium hydroxide, 1 g silver nitrate semi-dissolved in 5 ml distilled water, added drop wise) for 5 minutes before being washed 3 × 10 minutes in distilled water. 50 ml of developer (100 ml distilled water, 50 µl formaldehyde, 50 µl citric acid [100 mg.ml⁻¹]) was added for each gel. Once the bands had sufficiently developed on the gel, stop solution was added (0.5% [v/v] acetic acid) for no more than a minute. The gels could be stored for a few hours in distilled water. For longer term storage, the gels were dried for 1 hour at 80°C (Bio-Rad model 583 gel-drier with Welch 1426 GelMaster™).

For alcian blue silver staining, the gel was fixed overnight in alcian blue fixative (0.05% [w/v] alcian blue 8GX (Sigma, A5268) in fixative solution. The procedure was then identical to sodium-meta-periodate silver staining, excluding the oxidation with sodium-meta-periodate.

2.9.7 Visualising DOC-PAGE by silver staining

The staining of DOC-PAGE gels used the same solutions as SDS-PAGE staining (method 2.9.6), the only difference being the length of washes and incubations. For sodium-meta-periodate silver staining, the LPS gels were incubated overnight in fixative solution. To visualise sugar residues, the gel was covered with 50 ml of oxidiser. After 10 minutes, the gel was washed 5×5 minutes in distilled water. The gels were silver stained for 10 minutes before being washed 4×5 minutes in distilled water. 50 ml of developer was added for each gel. Once the bands had sufficiently developed on the gel, stop solution was added for no more than a minute. The gels could be stored for a few hours in distilled water. For longer term storage, the gels were dried for 1 hour at 80°C (Bio-Rad model 583 gel-drier with Welch 1426 GelMaster™).

For alcian blue silver staining, the gel was fixed overnight in alcian blue fixative. The procedure was then identical to sodium-meta-periodate silver staining, excluding the oxidation with sodium-meta-periodate.

2.9.8 LPS analysis by HPLC

Samples were analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) in a carbohydrate analyser system (Dionex), equipped with a ED50 electrochemical detector with gold electrode, a GS50 pump gradient, and a CarboPac PA200 analytical column (3 x 250 mm) with a CarboPac PA200 guard column (3 x 50 mm). To elute the samples, an isocratic gradient of 3.2 mM NaOH with a flux rate of $0.15 \text{ ml} \cdot \text{min}^{-1}$ was applied for 20 minutes. The column was washed with 200 mM NaOH and equilibrated with 3.2 mM NaOH prior to the next analysis. Polysaccharide samples (1 mg) were resuspended in 50 mM Sodium Acetate (pH 5.2) and 20 μl of sample was injected into the HPLC. Polysaccharide samples digested with Cellulase had 10 units of Cellulase (Trichoderma Viride, Sigma) added and incubated at 37°C for 1 hour. Samples were stored at 4°C until analysed.

2.10 Cloning and expression

2.10.1 Polymerase Chain Reaction (PCR)

To amplify the required genes from *P. profundum* SS9R, primers were designed containing restriction enzyme sites to allow directional cloning into a vector (Table 8). *pbpra1210* primers are designed for cloning of *pbpra1210* into the Qiagen pQE30 IPTG inducible expression plasmid. *pbpra0218* and *pbpra2686* were designed for complementation of the FL26 and FL9, respectively, using the arabinose inducible vector pFL190. Using pFL190, the primer sequences also had to include the upstream Ribosomal Binding Site (RBS).

Table 8: Primer Sequences

Primer	Sequence (5' – 3') ^{a b}
pbpra1210-F_SacI	ATGAGCT/CAATAACAGTTATCCAAAAGGTAGC
pbpra1210-R_XmaI	ATC/CCGGGCTATTTGTTAATAAGCTCATTGCC
pbpra0218-22F_EcoRI	ATG/AATTCAGAGAATATTTTAAACTACTGATGT
pbpra0218-R_XbaI	ATT/CTAGATTAGCAATTGTTCTTCTTTGAAGTT
pbpra0218-0217R_XbaI	ATT/CTAGATTATTTCTTTTTTTTGTAGATTGAAAA
pbpra2686-16F_BsaI	ATATATGGTCTCG/AATTCTAGTTGAAAAACATTTATGAGCAAC
pbpra2686-R_XbaI	ATT/CTAGATTATTCATAGCTATAATTATAATATCC
pbpra2686_714FSequence	AGGTAGAACTTCAGGTATTATGCAGCTTAC
pbpra2686_1134FSequence	GCGGTTAACAAGAGATGTAAATGTAAATCA
pFL190-F	GCGGGACCAAAGCCATGACAAAAA
pFL190-R	TTGTAAAACGACGGCCAGTGAGC

^a The italicised and underlined basepairs indicate the restriction enzyme recognition site

^b The slash indicates the restriction enzyme cleavage site

PCR Reaction mixtures:

Mix 1: 1 µl forward primer, 1 µl reverse primer, 2 µl 10⁻¹ purified plasmid, 6 µl distilled water.

Mix 2: 1 µl 10 mM 4x dNTP, 2 µl 10x polymerase buffer, 6.5 µl distilled water, 0.5 µl polymerase.

Mixtures 1 and 2 were added together 1:1 and the PCR reaction was performed as follows (Eppendorf MastercyclerES EP gradient S).

Table 9: PCR procedure

Step	Temperature (°C)	Time (minutes: seconds)
1) Lysis	94	4: 00
2) Denaturation	94	1: 00
3) Annealing	X	0: 50
4) Extension	72	Y
Repeat steps 2 - 4 34 times		

X – annealing temperature varied depending on length of primers

Y – extension time varied depending on length of amplicon, generally 1kb per minute

The PCR products were then visualised by agarose gel electrophoresis.

2.10.2 Agarose gel electrophoresis

Purified DNA or PCR products were analysed using agarose gel electrophoresis using 0.8, 1 or 1.3% w/v agarose (Lonza Seakem[®] LE agarose 50004) in 1x TAE buffer (50× TAE buffer [242 g.l⁻¹ Tris, 18.61 g.l⁻¹ Na₂EDTA·2H₂O, 57.1 ml Acetic acid]). 3 µl loading buffer (30% [v/v] glycerol, 0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol FF) was added to 5 µl of the purified DNA, and 6 µl loading buffer was added to 20 µl of PCR product. 8 µl of the sample/buffer mix was then loaded into the gel. The control ladder used was either New England Biolabs 1 kb (N32325) or 100 bp DNA ladder (N32315). 2 µl of the ladder was added to 10 µl distilled water and 2 µl loading buffer. 6 µl of the ladder mix was loaded onto either side of the gel. The gel was run at 80 V for 40 – 50 minutes for 0.8 and 1% gels and 1.5 hours for 1.3% gels. Where required, DNA fragments/plasmids were recovered from agarose gel using QIAquick gel extraction kit (Qiagen 28706).

2.10.3 Plasmid purifications

Plasmids were isolated from 5 ml overnight *E. coli* cultures using Qiaprep Spin Miniprep kit (Qiagen) following manufacturer's instructions. The plasmid was eluted into 50 µl elution buffer and stored at -20°C.

2.10.4 Restriction digests

P. profundum gene sequences and pQE30 were sequentially digested with the required restriction enzymes following manufacturers guidelines, heat inactivating the enzymes at 65°C for 20 minutes afterwards. In brief, digests were prepared as follows: DNA 8 µl, enzyme buffer, 2 µl, BSA, 0.5 µl, 7.5 µl distilled water, enzymes 1 µl each. *EcoRI*, *XbaI*, *SmaI* and *XmaI* were all incubated at 37°C for 1 hour (*XbaI* for 2 hours), *BsaI* was incubated at 50°C for 2 hours.

2.10.5 Ligation

Typically, a 4:1 ratio of ng insert to ng vector was used, 1 µl 10× ligase buffer, 1 µl T4 DNA ligase (Promega) and made up to 10 µl with distilled water. A vector alone reaction was also performed as a control. The mixtures were incubated at 4°C overnight before being transformed into competent *E. coli* the following day.

2.10.6 Preparation of competent cells

In order to create competent *E. coli*, a culture was first incubated overnight at 37°C with the necessary antibiotics in LB. This was then diluted to OD₆₀₀ of 0.5 in 10 ml LB and incubated at 37°C for 2 hours. The cells were harvested by centrifugation (Heraeus Labofuge 400R, 8179 rotor) and resuspended in 1 ml cold 100 mM CaCl₂ and incubated at 4°C for 1 hour. The cells were again pelleted by centrifugation and resuspended in 0.5 ml cold 100 mM CaCl₂. 50 µl aliquots of this suspension were stored at -80°C until needed.

2.10.7 Transformation of *E. coli*

5 µl plasmid (with/without insert) was added to 50 µl competent *E. coli*. This was incubated on ice for 30 minutes before being heated to 42°C for 2 minutes. The mixture was then transferred to 1 ml LB without antibiotics and incubated at 37°C for

1 hour. The cells were harvested by centrifugation and resuspended in 200 μ l LB and spread onto an LB agar plate containing the required antibiotics and incubated at 37°C. For this experiment 3 transformations are required, control untransformed *E. coli*, *E. coli* transformed with ligation control mixture (double digested vector alone), and *E. coli* transformed with ligation mixture (double digested vector and double digested insert).

Plasmids were typically transformed into *E. coli* DH5 α to maintain the plasmid in a stock containing no other plasmids.

2.10.8 Quick Transformation of *Salmonellae*

A 5 ml culture was grown to stationary phase, then diluted to an OD₆₀₀ of 0.1 and incubated at 37°C 200 rpm until the OD₆₀₀ was 0.6. 1.5 ml of this was pelleted and resuspended in 500 μ l cold 50 mM CaCl₂ and incubated on ice for 10 minutes. This was pelleted again and resuspended in 300 μ l cold 50 mM CaCl₂ and incubated on ice for 30 minutes. The cells were split into 50 μ l aliquots and 5 μ l of DNA was added and incubated on ice for 30 minutes before being heat shocked at 42°C for 2 minutes. The cells were recovered by adding 1 ml LB and incubated at 37°C 200 rpm for 1 hour. The cells were then pelleted and resuspended in 100 μ l fresh LB and spread onto LB agar plates containing the required antibiotic.

2.10.9 Screening transformants by PCR and restriction digest

Transformants were either screened by PCR, whereby a PCR product of the correct size would indicate the gene was successfully cloned into the vector; or by restriction digest using the appropriate restriction enzymes again looking for a product of the correct size.

2.10.10 Screening transformants by colony blot

Transformants that produced a His-tagged protein were also screened using the colony blot procedure (QIAexpressionist manual, protocol 4, QIAGEN) (*pbpra1210* only). In brief, transformants were purified to single colonies. A single colony from each transformant was removed onto a fresh LB agar plate containing the relevant antibiotics, so that the plate contained all the transformants in a grid like pattern. Onto this plate the positive and negative controls could be added if they had the same antibiotic resistance. In this experiment the negative controls were *E. coli* M15 pREP4 and *E. coli* M15 pREP4 pQE30, only the latter containing pQE30 could be grown on the same plate because pQE30 provides Ampicillin resistance. *E. coli* M15 pREP4 was grown on LB agar containing Kanamycin. The positive control also required a separate LB agar plate containing only Ampicillin, this was *E. coli* BL21 231A (*E. coli* BL21 containing pETDEST42 + *cysK*). This plasmid vector also labels proteins with a His tag. The plates were incubated overnight at 37°C before being transferred to a nitrocellulose membrane. The membrane was placed colony side up onto fresh LB agar plates containing the required antibiotics and 250 µM IPTG. The cultures were then incubated at 37°C for 4 hours before the nitrocellulose membranes were treated to lyse the cells (QIAexpressionist manual, protocol 4). The membrane was visualised using the Ni-NTA-HRP conjugate (QIAGEN) and homemade chemiluminescent ECL solutions (see method 2.10.14).

2.10.11 Mating into *P. profundum* SS9

Experiments have shown that transformation of *P. profundum* is unsuccessful. It was therefore necessary to mate the complementing plasmids in using a helper plasmid, pRK2013. The *P. profundum* strain was grown in 5 ml marine broth (no antibiotics) for 48 hours (15°C and 0.1 MPa). The culture was diluted 1:50 in fresh marine broth and grown overnight. Each mating required 5 ml *P. profundum* culture.

The *E. coli* donor strain (DH5α strain containing pFL190 with complementing gene) was grown overnight in LB + streptomycin (100 µg.ml⁻¹), and the *E. coli* helper

CSH56 pRK2013 in LB + kanamycin ($50 \mu\text{g.ml}^{-1}$). The *E. coli* cultures were washed once with an equal volume of marine broth. All cultures were pelleted by centrifugation (10 min at 4500 rpm and 15°C) and resuspended in fresh marine broth, *P. profundum* in $100 \mu\text{l}$ marine broth and *E. coli* cultures in 1 ml marine broth. A 1:1:1 ratio ($75 \mu\text{l}$ each) of these cell suspensions were mixed together and the total volume ($225 \mu\text{l}$) plated directly onto a marine agar plate. The plates were incubated immediately (i.e. without allowing mixture to dry onto plate) in the dark at 20°C and 0.1 MPa for 40 hours. Cells were scraped from the agar plates with a sterile plastic loop and resuspended in 1 ml of marine broth. $100 \mu\text{l}$ of this mixture was spread onto a marine agar containing streptomycin ($150 \mu\text{g.ml}^{-1}$) and rifampicin ($100 \mu\text{g.ml}^{-1}$) and incubated at 15°C and 0.1MPa. After 10 days isolated colonies were purified onto marine agar containing rifampicin ($100 \mu\text{g.ml}^{-1}$) and streptomycin ($150 \mu\text{g.ml}^{-1}$) and incubated at 15°C and 0.1MPa. Colonies were purified for a second time before being using to inoculate 5 ml marine broth with streptomycin ($100 \mu\text{g.ml}^{-1}$) and incubated at 15°C 0.1 MPa for 48 hours.

2.10.12 Induction and purification

E. coli M15 pREP4 containing the expression vector pQE30 and *pbpra1210* insert were grown to log phase and induced by addition of 1 mM IPTG following QIAexpressionist[™] protocol 5, (QIAGEN). The cells were harvested by centrifugation at 3500 rpm for 20 minutes and resuspended in lysis buffer for native purification. 1 mg.ml^{-1} lysozyme was added to each sample and incubated on ice for 30 minutes before sonicating at 10 amplitude microns for 10 seconds with 10 second pauses, repeating 6 times. The mixtures were centrifuged at 9000 g for 30 minutes, the supernatant represented to soluble fraction and the pellet the insoluble fraction. The pellet was resuspended in lysis buffer for native purification.

The purification under denaturing conditions protocol published in QIAGEN QIAexpressionist (Fourth Edition, January 2000, Protocol 10) was followed in order to solubilise insoluble proteins. The insoluble cell pellet was resuspended in denaturing

buffer (containing 8M Urea) and mixed frequently at room temperature, after the solution turned translucent (~ 1 hour). The mixtures were recentrifuged at 9000 g for 30 minutes and the supernatant now represented the soluble fraction and was analysed as previously discussed.

2.10.13 Analysis of Protein expression by SDS-PAGE

Buffers made according to QIAGEN QIAexpressionist™ (Fourth Edition, January 2000, Protocol 5).

Table 10: Buffers required for SDS-PAGE of proteins

Separating Gel	8% (ml)	10% (ml)	14% (ml)
distilled water	6.3 ml	5.05	2.55
1M tris pH 8.8	7.05	7.05	7.05
10% (w/v) SDS	0.188	0.188	0.188
30% (w/v) acrylamide, 0.8% (w/v) bis acrylamide sol ⁿ	5	6.25	8.75
10% (w/v) APS	0.188 (188 µl)	0.188 (188 µl)	0.188 (188 µl)
TEMED	0.01 (10 µl)	0.01 (10 µl)	0.01 (10 µl)
Concentrating Gel	4% (ml)		
distilled water	5.4		
1M tris pH 6.8	0.950		
10% (w/v) SDS	0.075 (75 µl)		
30% (w/v) acrylamide, 0.8% (w/v) bis acrylamide sol ⁿ	1		
10% (w/v) APS	0.075 (75 µl)		
TEMED	0.015 (15 µl)		

Samples mixed 1:1 with sample buffer (QIAGEN QIAexpressionist™ (Fourth Edition, January 2000, Protocol 5) and were then heated to 95°C for 5 minutes. 10 µl of each sample was loaded onto gels. Gels were run at 80 V for the concentrating gel, then 150 V for the separating gel. Gels were stained for 1 hour with coomassie (2.5 g coomassie brilliant blue R-250, 450 ml methanol, 100 ml acetic acid, made to 1 litre with distilled water and filter sterilised) and destained overnight in distilled water.

2.10.14 Analysis of proteins by Western blot

The gel, Highbond ECL membrane (Amersham biosciences) and 4 pieces of blotting paper were soaked in 1x Transfer buffer (10x Transfer buffer [250 mM Tris base, 1.5 M glycine], 1x Transfer buffer [100 ml 10x Transfer buffer, 3.75 ml 10% (w/v) SDS, 200 ml methanol, made to 1 L with distilled water]). 2 pieces of blotting paper were placed into the semi-dry transfer system (Bio-Rad), covered with the ECL membrane, then the gel, and the remaining 2 blotting paper pieces on top. The proteins were transferred at 15 V (60 minutes) in the cold room. After transfer, the membrane was incubated overnight at 4°C shaking in blocking buffer (5% [w/v] milk powder, 0.25% [v/v] Tween 20, make to 100 ml with 1x PBS). The membrane was then washed 4 × 5 minutes in wash buffer (0.05% [v/v] Tween 20 in 1× PBS) and the His-tagged protein was detected using detection solution (0.05% [v/v] Tween 20, 1% [w/v] milk powder, 1/2000 QIAGEN Ni-NTA-HRP conjugate, make to 10 ml in 1× PBS) for 2 hours at room temperature. The proteins were visualised by adding ECL solutions A (1 ml 250 mM luminol, 440 µl 90 mM *p*-coumaric acid, 10 ml 1 M Tris-HCl pH 8.5, made to 100 ml with distilled water) and B (64 µl 8.8 M H₂O₂ (30% [w/w]), 10 ml 1 M Tris-HCl pH 8.5, made to 100 ml with distilled water)) together 1:1 and expose for at least 1 minute to autoradiography film or a dark room camera.

3. Results - The effects of temperature and pressure changes on the physiology of *P. profundum* SS9R

3.1 Introduction

Photobacterium profundum SS9 is a piezophilic and psychrophilic bacterium that can grow over a range of pressures (0.1–90 MPa) and temperatures (2–20°C) (DeLong, 1986). Since being isolated, genetic and proteomic approaches have given insights into *P. profundum* SS9 regulatory systems at pressure (Bartlett and Welch, 1995; Bidle and Bartlett, 1999; Vezzi *et al.*, 2005; Welch and Bartlett, 1996). The fatty acid composition of *P. profundum* SS9 has also been demonstrated to change with growth at cold temperatures and high pressures (Allen *et al.*, 1999). Additionally, cis-vaccenic acid (C18:1) was found to be essential for optimal growth of *P. profundum* SS9 under cold temperatures and high pressures (Allen and Bartlett, 2000). The membranes of bacteria have evolved to adapt to environmental conditions, such as the total cell fatty acid composition in *E. coli* is known to be regulated in response to temperature, which maintains the fluidity of the membrane by introducing longer chain fatty acids, branched fatty acids and higher levels of unsaturated fatty acids (Marr and Ingraham, 1962; Sinensky, 1971). Gram negative bacteria are characterised by the structure of the cell envelope, specifically having an inner and outer membrane. The outermost part of the outer membrane consists of lipopolysaccharides (LPS) and, in some cases, capsular polysaccharides (CPS). The LPS is typically a heterogeneous mixture of different lengths of O-antigen, which leads to the characteristic laddering profile of bacteria such as *Salmonella* sp. when visualised by PAGE. The shortest form of LPS is called rough LPS and the longest is smooth LPS, so called because of the appearance of colonies grown on agar plates. The LPS structure is important because alterations have been shown to affect the sensitivity of bacteria to certain stresses (Bennett *et al.*, 1981; Thomsen *et al.*, 2003). The biosynthesis of CPS has been well characterised in *E. coli* and *Salmonella*, however the attachment of CPS to the outer membrane is still under debate. While CPS is postulated to be covalently linked to the outer membrane, no

linker has yet been discovered. In *E. coli*, a mutation in *wzi* leads to a dissociated CPS, however the CPS is known not to be attached to Wzi (Rahn *et al.*, 2003).

The LPS of mesophilic bacteria has been shown to adapt in response to temperature changes, for example, *Salmonella enterica* serovar Minnesota alters its Lipid-A content to contain significantly more C16:1 and C18:1 at 12°C than at 37°C (Wollenweber *et al.*, 1983). This was accompanied by a reciprocal decrease in saturated fatty acids. Psychrophilic bacteria have also been shown to modify their LPS composition at cold temperatures. The LPS of *Pseudomonas syringae* has a reduced amount of phosphorylation at low temperatures (Ray *et al.*, 1994). *P. syringae* has also been shown to incorporate a greater amount of hydroxylated fatty acids into its LPS at low temperatures (Kumar *et al.*, 2002). The changes in membrane composition are believed to help maintain the fluidity of the membrane, called homeoviscous adaptation (Sinensky, 1974). *P. profundum* SS9 has also been demonstrated to modify the total cell fatty acid composition in response to temperature and pressure (Allen *et al.*, 1999; Allen and Bartlett, 2000). In contrast to cold temperatures, the effects of high pressures on LPS composition has not yet been fully investigated.

This chapter describes the development of methodologies used to characterise *P. profundum* SS9R, a rifampicin resistant derivative of SS9 (Chi and Bartlett, 1993). Additionally, the growth and morphology of cells from cold temperatures and high pressures, and the expression and composition of LPS were also investigated.

3.2 Characterisation of growth under different conditions

Prior to undertaking this research, there has been little physiological characterisation of *P. profundum* SS9. Therefore, some basic analysis of the growth and behaviour of *P. profundum* SS9R under different conditions was performed.

3.2.1 Growth of *P. profundum* SS9R in marine broth

The growth of *P. profundum* SS9 required meticulous preparation of marine broth (Difco 2216). During preparation the broth contains a large amount of precipitate, which was found to detrimentally affect the growth of *P. profundum* SS9 in both marine broth and agar (data not shown). The marine broth was therefore filtered to remove the precipitate. To the marine broth, a buffer was added to maintain the pH of the solution. Without a buffer, the marine broth became acidic after 48 hours of growth, resulting in the lysis of the culture (data not shown). Finally, glucose was added to the marine broth. While not essential for aerobic cultures, glucose was absolutely required for anaerobic growth.

P. profundum SS9 has been shown to be capable of growth over a range of temperatures (2-20°C) (DeLong, 1986). Before investigating other factors, the growth of *P. profundum* SS9R was compared at 15 and 4°C (0.1 MPa). A culture was diluted to OD₆₀₀ 0.1 and heat sealed in sterile plastic Pasteur pipettes. At each time point a single Pasteur was used to measure OD₆₀₀ and for colony forming units (cfu). The growth at 15°C was faster than at 4°C (Figure 11A and B, respectively) which supports the finding that 15°C has been previously shown to be the optimum growth temperature for *P. profundum* SS9 (DeLong, 1986). Both the 15 and 4°C growth curves show a steady increase in OD₆₀₀, with entry into stationary phase at 30 hours for 15°C and 110 hours for 4°C and reach a maximum OD₆₀₀ of just over 1. However, the cfu data was significantly more variable, especially for the 4°C cultures (Figure 11C and D). Both 15 and 4°C cfu data showed large changes in cell number, up to as much as 1000 fold difference. Given the variability of the cfu data, the OD₆₀₀ was used as a measure of the

growth of *P. profundum* SS9 cultures. As demonstrated in later sections and figures, such as Figure 13, the OD₆₀₀ was a reliable measure because cultures diluted to a required OD₆₀₀ would invariably result in comparable growth. For example, cultures diluted to OD₆₀₀ 0.2 reproducibly showed growth to 10⁻⁴ dilution. The variability found in cfu data compared to the OD₆₀₀ data could be due to the experimental procedure used. The cfu variability is greater in the 4°C culture and this may relate to the recovery of the cells for the cfu measurements, being incubated at 4°C and recovered at 15°C.

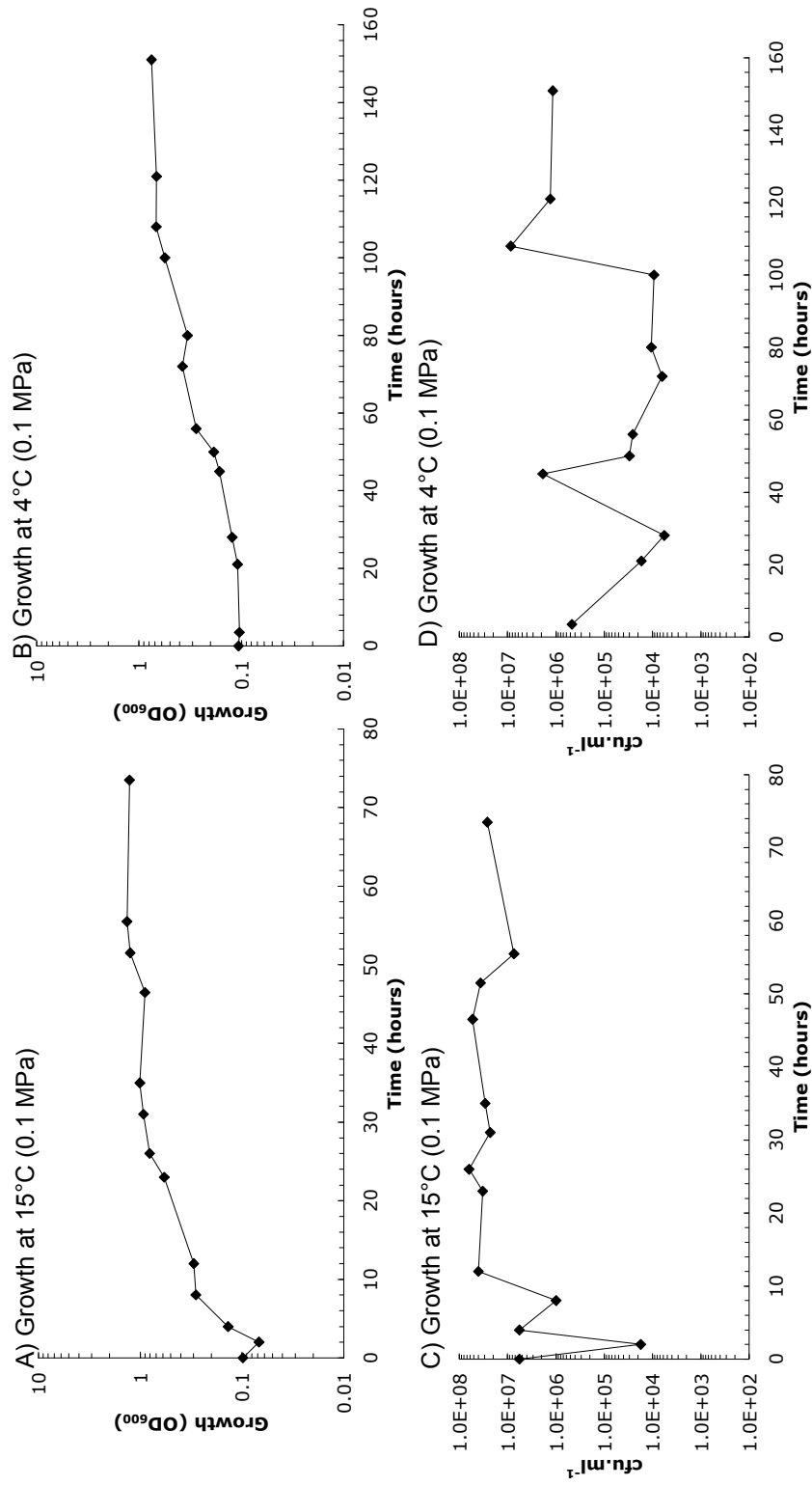


Figure 11: Colony forming units (cfu) are an unreliable measure of growth for *P. profundum* SS9R

A stationary phase culture of *P. profundum* SS9R from 15°C (0.1 MPa, anaerobic) was diluted to a starting OD₆₀₀ of 0.1 in fresh marine broth and heat sealed in sterile plastic Pasteur pipettes (anaerobic). These pipettes were incubated as defined. The optical density was measured to assess growth (A and B) and an aliquot from each time point was serially diluted to 10⁻⁶. These dilutions were pipetted onto marine agar plates in triplicate spots of 10 µl each. The plates were incubated at 15°C (0.1 MPa) for up to 5 days and the colonies counted (C and D).

P. profundum SS9R was characterised as a piezophilic bacterium (Allen *et al.*, 1999; Bidle and Bartlett, 1999; DeLong, 1986). Therefore, the effect of pressure on the growth of *P. profundum* SS9R in liquid culture was investigated. Cultures grown at 0.1 MPa (15°C) had an extended lag phase compared to growth at 28 MPa (15°C) and a reduced exponential growth rate (Figure 12A). These differences in the growth at 28 and 0.1 MPa (15°C) demonstrate the piezophilic nature of *P. profundum* SS9R, which will grow optimally at 28 MPa (15°C) (DeLong, 1986).

In contrast to non pressure-adapted bacterial species (Zobell and Cobet, 1962), *P. profundum* SS9R filaments at 0.1 MPa and remains rod shaped at 28 MPa with an average length of between 2 - 3 µm (Figure 12B and C, respectively). The cells at atmospheric pressure were not exclusively filamentous cells, these were only a part of the population at 0.1 MPa, with the majority being made up of various pleomorphic shapes and enlarged/swollen rods. Also the morphology of the cells was dependent on the growth phase. Whilst cultures grown at 0.1 MPa were filamentous and pleomorphic during log phase, stationary phase cells were enlarged and swollen (compare Figure 12B with D). 28 MPa cultures during log phase were almost exclusively dividing (hence the increased length), whereas during stationary phase the doubling rate has significantly reduced and single cells were more easily distinguished (compare Figure 12C with E).

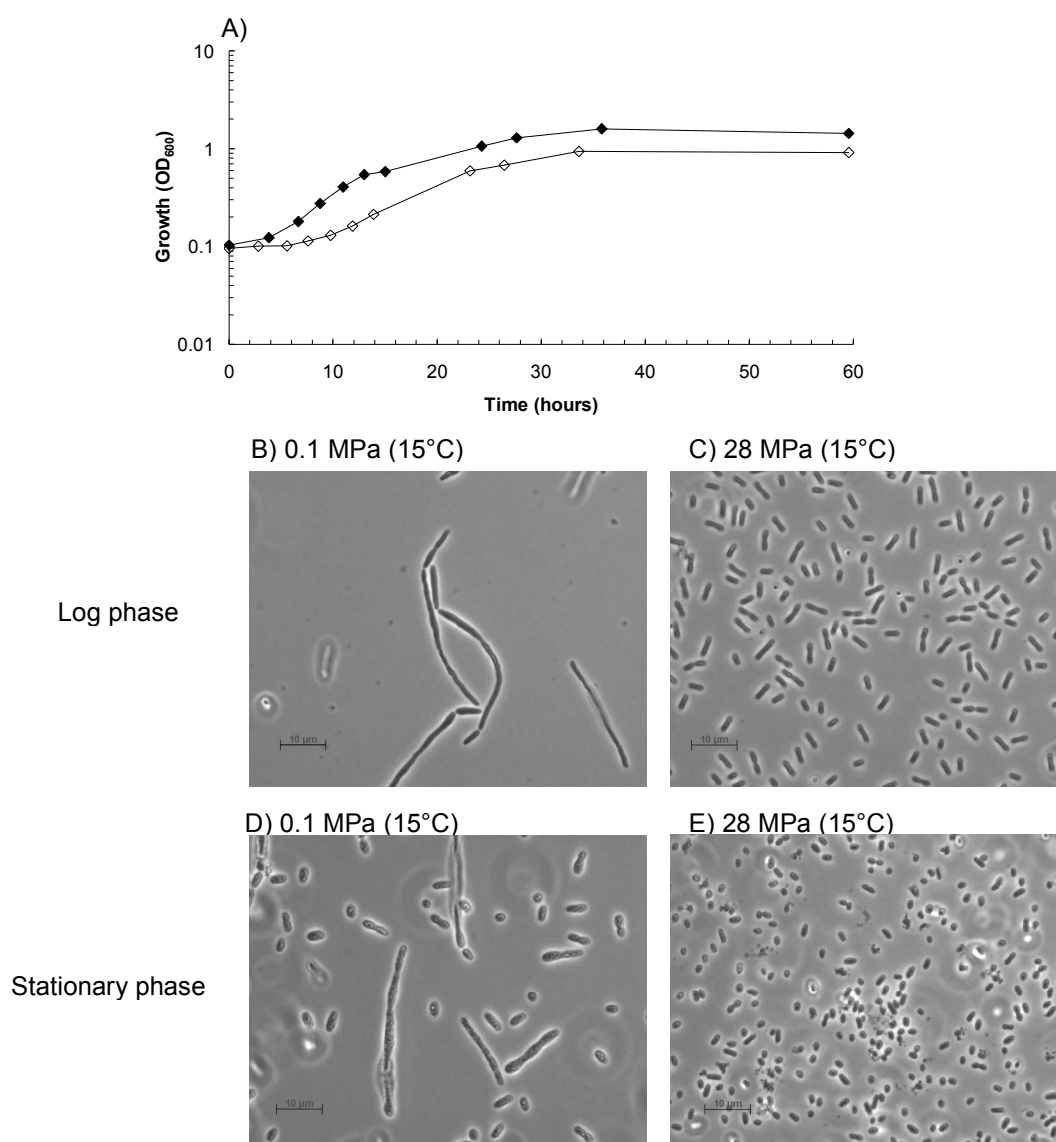


Figure 12: *P. profundum* SS9R has better growth at 28 MPa 15°C than 0.1 MPa 15°C

A stationary phase culture of *P. profundum* SS9R from 15°C (0.1 MPa, anaerobic) was diluted to a starting OD₆₀₀ of 0.1 in fresh marine broth and heat sealed in sterile plastic Pasteur pipettes (anaerobic). These pipettes were incubated at 0.1 MPa (15°C) (◇) and 28 MPa (◆) (15°C). Images above were taken during log phase after 10 hours of incubation (B and C) and stationary phase after 30 hours of incubation (D and E). Bar equals 10 µm.

3.2.2 *P. profundum* SS9R growth on marine agar

The growth of *P. profundum* SS9R at cold temperatures was also examined on marine agar plates (Figure 13). Some stresses can be perceived differently depending on the environment the bacteria are encountering, such as cold shock in *E. coli* K12 (Frédérique Perrot, 2001). The protein expression profile of *E. coli* K12 has been shown to be different under cold shock depending on whether the cells were immobilised or in liquid culture. *P. profundum* SS9R was incubated on plates at 15, 8, 4 and 2°C (0.1 MPa). After 4 days incubation the plate at 15°C showed growth until 10^{-4} dilution. The lower temperatures resulted in slower growth. At 8 and 4°C there was significant growth until 10^{-2} dilution, and at 2°C there was no significant growth. Nevertheless after 10 days incubation the growth at 8 and 4°C were similar to that at 15°C, with growth on dilutions up to 10^{-4} . The plates incubated at 2°C required an extended incubation to reach 10^{-4} dilution, which took 25 days in total. Importantly, *P. profundum* SS9R had significant growth to the same dilution at all temperatures tested. Smaller colonies in the cold temperatures was a result of reduced growth rate and extended incubation eventually resulted in colony sizes equivalent to those at 15°C.

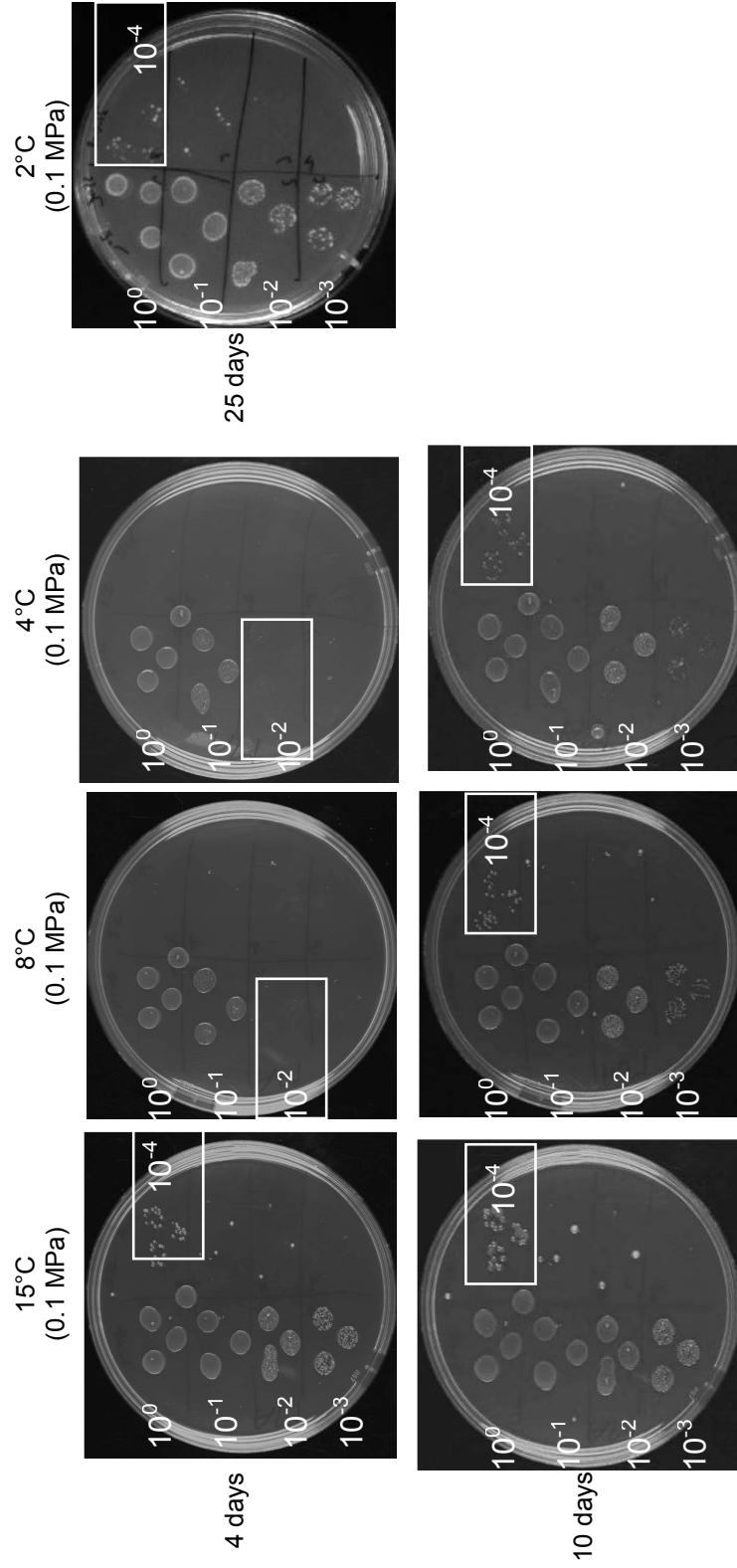


Figure 13: *P. profundum* SS9R has comparable growth at 15 and 4°C

An anaerobic stationary phase culture of *P. profundum* SS9R from 15°C (0.1 MPa) was diluted to OD_{600} 0.2 in fresh marine broth and serially diluted to 10^{-6} . These dilutions were spotted in triplicates of 10 μ l each onto marine agar plates containing 20 mM glucose. The plates were incubated as defined. White boxes highlight the lowest dilution with significant growth.

3.2.3 A method for assessing growth of *P. profundum* SS9R on solid agar at high pressures

As the cfu could not be reliably used to assess the growth with *P. profundum* SS9R (Figure 11), a method of assessing growth under high pressures on solid agar was designed which was modified from a published method (Masui and Kato, 1999). In this method cultures were spotted onto marine agar plates (similar to Figure 13) and overlaid with molten agar. The plates were then removed from the petri dish and sealed in a sterile plastic pouch. Attempts to reproduce the published method were unsuccessful because the marine agar plates disintegrated when incubated at 28 MPa (15°C) (data not shown). To avoid heat stressing the bacteria with molten agar, a piece of solidified marine agar was placed on top of the spotted plate. Although this removed the heat stress of molten agar, it also prevented the growth of defined single colonies because the top and lower plates were incompletely immobilised. To reduce heat stress as far as possible, the standard agar was replaced with a low gelling temperature alternative called Low Temperature Gelling Marine Agar (LTGMA). In addition, the LTGMA was also found to be less likely to disintegrate than the usual marine agar after incubation at pressure (data not shown).

To ensure that the growth of *P. profundum* SS9R was the same on both marine agar and LTGMA, the growth was compared. Significant growth was seen on both types of media until 10^{-4} dilution (Figure 14A and B) which demonstrated that the LTGMA did not affect growth of *P. profundum* SS9R. Overlaying with molten LTGMA reduced the growth of *P. profundum* SS9R at 0.1 MPa (15°C) to 10^{-1} dilution compared to the non-overlaid LTGMA which showed that molten LTGMA was harmful to *P. profundum* SS9R (Figure 14C and B, respectively). Overlaid LTGMA plates incubated at 28 MPa (15°C) had significant growth until 10^{-4} dilution, more than when incubated at 0.1 MPa (15°C) (compare Figure 14D with C). While overlaying with molten LTGMA was detrimental to *P. profundum* SS9R, increased growth was observed at 28 MPa (15°C) compared to 0.1 MPa (15°C), which showed that incubation of overlaid plates under optimal growth conditions for *P. profundum* SS9R allowed the

recovery of a greater number of bacteria than at 0.1 MPa. Therefore, this method could be used to assess high pressure-adapted growth on solid agar.

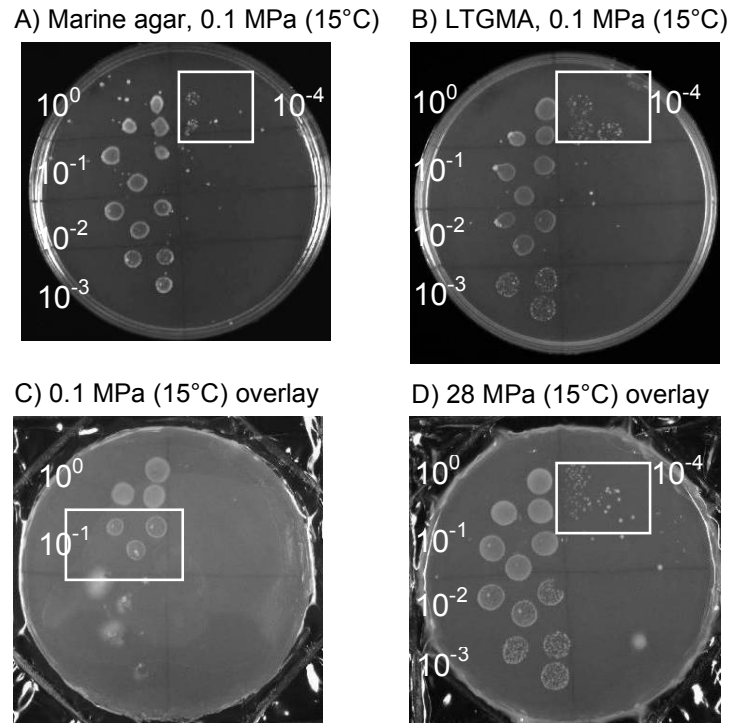


Figure 14: *P. profundum* SS9R grows better at 28 MPa than 0.1 MPa (15°C)

A stationary phase cultures of *P. profundum* SS9R from 15°C (0.1 MPa, anaerobic) was diluted to an OD₆₀₀ of 0.8 in fresh marine broth and serially diluted to 10^{-6} . These dilutions were spotted in triplicates of 10 µl each onto marine agar (A) and low temperature gelling marine agar (LTGMA) (B-D) plates. 2 LTGMA plates were incubated at 0.1 MPa (15°C) for 2 hours before being overlaid with molten LTGMA and sealed in sterile plastic bags (C and D). These plates were then incubated at 0.1 MPa (15°C) (A – C) and 28 MPa (15°C) (D). After 6 days the growth was assessed. White boxes highlight the lowest dilution with significant growth in all 3 spots.

3.3 The effects of temperature and pressure on LPS

P. profundum SS9 has been shown to modify the total cell fatty acid composition in response to temperature and pressure changes (Allen *et al.*, 1999; Allen and Bartlett, 2000). In contrast to lipids, little is known about the effects of pressure on LPS. The LPS of *P. profundum* SS9R grown at various temperatures and pressures was therefore analysed using various different methods.

3.3.1 SDS-lysis followed by SDS-PAGE does not show significant LPS alterations in response to temperature and pressure

The LPS was extracted from *P. profundum* SS9R stationary phase cultures by SDS-lysis and analysed by SDS-PAGE using two methods of silver staining. Sodium-m-periodate silver staining oxidises sugar residues (Dubray and Bezard, 1982; Tsai and Frasch, 1982) whereas alcian blue silver staining stains negatively charged or acidic residues (Corzo *et al.*, 1991). By using these two methods, it was found that each will stain different parts of the LPS profile. Sodium-m-periodate silver staining was more specific for the lower bands (likely the rough LPS) whilst alcian blue silver staining visualised the entire LPS profile (Figure 15A and B, respectively).

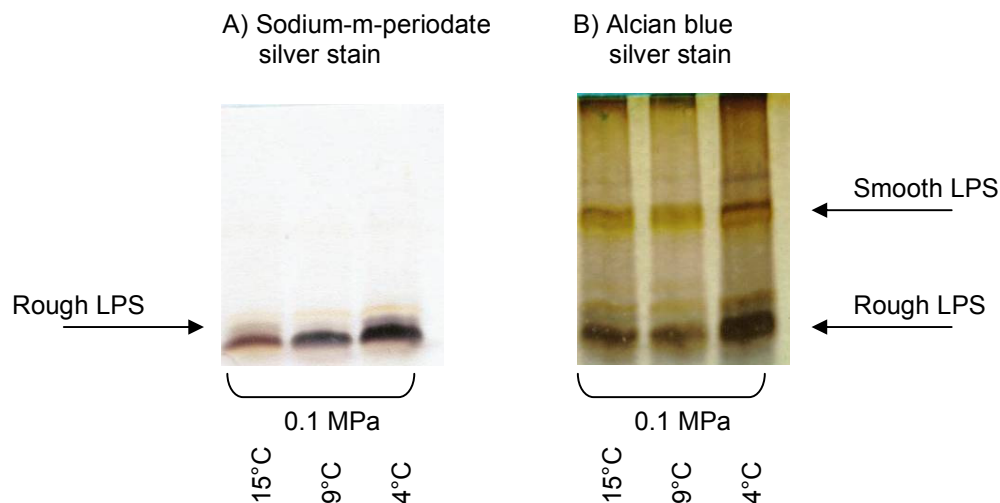


Figure 15: The SDS-PAGE LPS profile of *P. profundum* SS9R does not alter with temperature

A stationary phase culture of *P. profundum* SS9R from 15°C (0.1 MPa, anaerobic) was diluted in fresh marine broth, heat sealed in plastic Pasteur pipettes and incubated at 15°C, 9°C and 4°C (0.1 MPa, anaerobic). Stationary phase cultures were pelleted by centrifugation and the polysaccharides were isolated by SDS-lysis. The LPS was then analysed by SDS-PAGE followed by either sodium-m-periodate silver staining (A) or alcian blue silver staining (B).

3.3.2 Hot phenol water extraction followed by DOC-PAGE shows LPS alterations in response to temperature and pressure

Previous work has shown that different methods of extracting the LPS, such as the hot phenol water method (Carlson *et al.*, 1978; Darveau and Hancock, 1983; Westphal and Jann, 1965), can be more efficient for different species of bacteria. Hot phenol water extraction separates the LPS into aqueous and phenol phases according to its hydrophobicity.

LPS samples were taken from *P. profundum* SS9R grown at 15 and 4°C at both 0.1 and 28 MPa, extracted by hot phenol water and analysed by DOC-PAGE (Figure 16). Significantly more LPS was found in the aqueous phase than phenol phase

(data not shown). Aqueous phase LPS from cultures grown at 15°C (0.1 MPa) and 4°C (0.1 MPa) had a difference in the lower bands (Figure 16A). Aqueous phase LPS from cultures grown at 15°C (28 MPa) and 4°C (28 MPa) appeared identical. However, when comparing 0.1 MPa (15°C) and 28 MPa (15°C) aqueous phase LPS several lower bands were missing at 28 MPa. From the DOC-PAGE it looks as if *P. profundum* SS9R adapts its LPS in response to temperature changes at 0.1 MPa and in response to pressure at 15°C.

The phenol phase LPS only constitutes the lowest bands in the LPS profile, known as rough LPS (Figure 16B). This is because the rough LPS was separated into the phenol phase due to the increased hydrophobicity. The phenol phase LPS profiles only showed changes in response to pressure, not temperature.

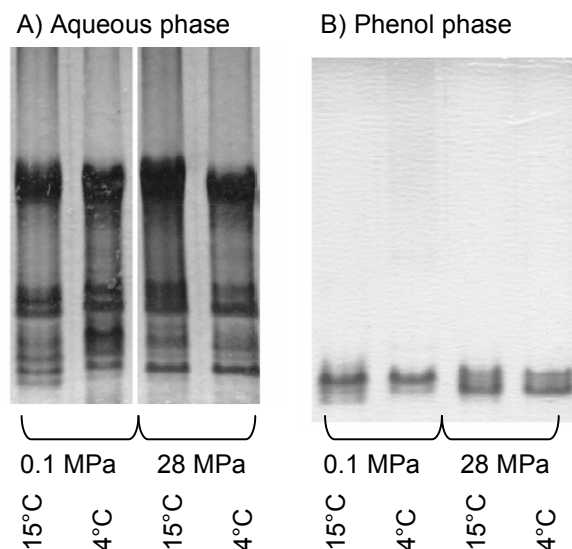


Figure 16: The DOC-PAGE LPS profile from *P. profundum* SS9R hot phenol water extraction alters with temperature and pressure

Stationary phase cultures from 15°C 0.1 MPa, 4°C 0.1 MPa, 15°C 28 MPa and 4°C 28 MPa were pelleted by centrifugation and the polysaccharide isolated by the hot phenol water method. The aqueous phase LPS (A) and phenol phase LPS (B) were then analysed by DOC-PAGE followed by alcian blue silver staining. Performed in collaboration with Dr. T. Tryfona.

3.3.3 Non-piezophilic bacteria do not have the same LPS alterations in response to temperature and pressure

Salmonella enterica serovar Typhimurium LT2 is a characteristic non-piezophilic bacterium that has been well studied, both in terms of its growth and the LPS biosynthesis and structure (Ames *et al.*, 1974; Bennett *et al.*, 1981; Osborn *et al.*, 1972). *S. Typhimurium* LT2 is therefore a useful comparison to *P. profundum* SS9. By comparing the effects of temperature and pressure on the LPS of *S. Typhimurium* LT2 and LPS biosynthesis mutants, it may be possible to gain insight into the likely mechanisms in *P. profundum* SS9.

P. profundum SS9 and *P. profundum* 3TCK are closely related *P. profundum* strains. While *P. profundum* SS9 is a moderate piezophile, *P. profundum* 3TCK is pressure sensitive. Therefore by studying the LPS of *P. profundum* 3TCK it is possible to distinguish whether the LPS alteration seen in *P. profundum* SS9 is related to pressure-adapted growth.

P. profundum 3TCK and *S. Typhimurium* LT2 grown anaerobically in sealed sterile Pasteur pipettes at 0.1 and 28 MPa (15°C for 3TCK, 37°C for LT2). While growth curves were not performed at this instance cultures under both pressure conditions had reached a high OD₆₀₀ (~2.0) after 18 hours incubation. The LPS of *P. profundum* 3TCK and *S. Typhimurium* LT2 were isolated by hot phenol water and analysed by DOC-PAGE sodium-m-periodate silver staining. *S. Typhimurium* LT2 has been demonstrated before to have a characteristic laddering pattern, which was reproduced here (Figure 17). The LPS of *P. profundum* 3TCK has not been previously analysed, and had a similar laddering pattern to that of the *S. Typhimurium* LT2 (Figure 17). *P. profundum* 3TCK had a similar separation of the LPS phases to *P. profundum* SS9R, with the phenol phase having significantly less LPS than the aqueous phase (data not shown). While the aqueous phase LPS from *S. Typhimurium* LT2 contained more LPS than the phenol phase, the phenol phase contained significantly more LPS than the *P. profundum* strains (data not shown). By this method, neither *P. profundum* 3TCK nor *S. Typhimurium* LT2 had a significant alteration in their LPS profiles when grown at 28 MPa compared to 0.1 MPa. This indicated that the piezophilic adaptation in

P. profundum SS9R involved the LPS and that these non-piezophilic bacteria do not share this adaptation.

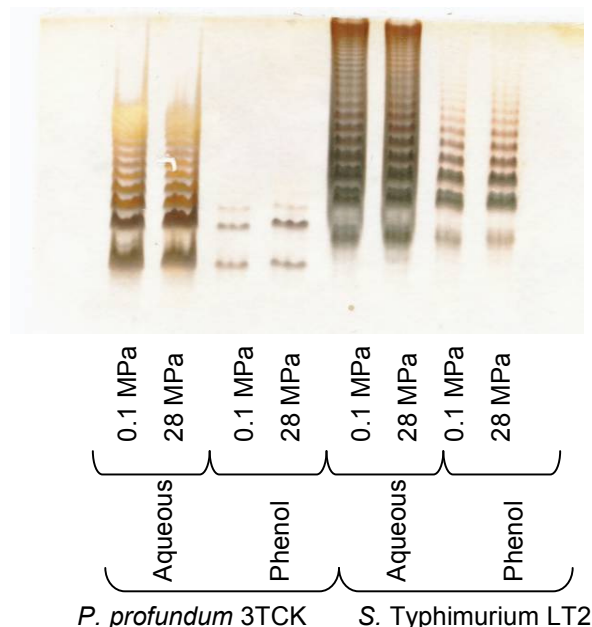


Figure 17: The DOC-PAGE LPS profiles of *P. profundum* 3TCK and *S. Typhimurium* LT2 do not alter when grown at 28 MPa

P. profundum 3TCK stationary phase cultures from 0.1 and 28 MPa (15°C) and *S. Typhimurium* LT2 stationary phase cultures from 0.1 and 28 MPa (37°C) were pelleted by centrifugation. The LPS was isolated by the small scale hot phenol water method and then analysed by DOC-PAGE followed by sodium-m-periodate silver staining.

The DOC-PAGE LPS profiles of *P. profundum* 3TCK and *S. Typhimurium* LT2 grown at different temperatures were also analysed for alterations (Figure 18). Neither *P. profundum* 3TCK nor *S. Typhimurium* LT2 had LPS profile alterations in response to growth at cold temperatures that were detectable by DOC-PAGE and silver staining. The LPS alterations visualised by DOC-PAGE in *P. profundum* SS9R in response to pressure and cold temperatures appear to be an adaptive feature not seen in other non-pressure adapted bacterial species.

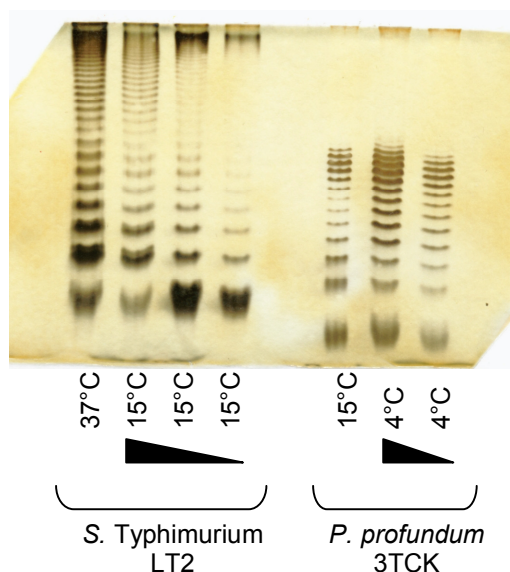


Figure 18: The DOC-PAGE LPS profiles of *P. profundum* 3TCK and *S. Typhimurium* LT2 do not alter when grown at cold temperatures

P. profundum 3TCK stationary phase cultures from 15 and 4°C (0.1 MPa) and *S. Typhimurium* LT2 stationary phase cultures from 37 and 15°C (0.1 MPa) were pelleted by centrifugation and the LPS isolated by the small scale hot phenol water method. The aqueous phase LPS were then analysed by DOC-PAGE followed by sodium-m-periodate silver staining. Dilutions from highest to lowest indicated by black right-angled triangle.

3.3.4 *P. profundum* SS9R LPS consists mainly of a glucan

To gain an insight into the LPS changes that occur in *P. profundum* SS9R when grown at cold temperatures and high pressures, compositional analyses were performed. *P. profundum* SS9R was grown anaerobically at 0.1 and 28 MPa (15°C), and 0.1 and 28 MPa (4°C). The aqueous phase LPS was extracted by the hot phenol water method and used to investigate fatty acid and carbohydrate composition. Compositional analysis was performed by the Complex Carbohydrate Research Centre (CCRC, Athens, Georgia, USA). Analysis of the fatty acid composition revealed that the LPS samples contained small amounts of fatty acids (Table 11). The only significant levels of fatty

acids were detected from cultures grown at 0.1 MPa, which included C12:O, C14:O, C16:O, 3-OH C14:O and C18:O. Of the cultures grown at 0.1 MPa, LPS from 15°C contained more C14:O, whilst LPS from 4°C contained more C16:O and C18:O. LPS from 4°C (0.1 MPa) whilst a greater amount of the longer chain fatty acids was interesting because longer chain fatty acids have been shown to maintain fluidity in the membrane (Sinensky, 1974). C12:O and C18:O were detected at 15 and 4°C (28 MPa) and C16:O was detected at 28 MPa (15°C). However these were only detected in trace amounts.

Table 11: Fatty acid analysis of *P. profundum* SS9R LPS grown under different conditions

	Temp/pressure of growth	0.1 MPa 15°C	28 MPa 15°C	0.1 MPa 4°C	28 MPa 4°C
Fatty Acid (% Mole*)					
<i>Decanoic Acid</i> (C10:O)		n.d.	n.d.	n.d.	n.d.
<i>Dodecanoic Acid</i> (C12:O)		33.7	Trace	34.1	Trace
<i>Tetradecanoic Acid</i> (C14:O)		15	n.d.	n.d.	n.d.
<i>Hexadecanoic Acid</i> (C16:O)		10.7	Trace	18	n.d.
<i>3 Hydroxytetradecanoic Acid</i> (3-OH C14:O)		33.2	n.d.	30	n.d.
<i>Octadecanoic Acid</i> (C18:O)		7.4	Trace	17.8	Trace
<i>3 Hydroxyhexadecanoic Acid</i> (3-OH C16:O)		n.d.	n.d.	n.d.	n.d.
<i>Hexadecenoic Acid</i> (C16:1)		n.d.	n.d.	n.d.	n.d.
<i>Octadecenoic Acid</i> (C18:1)		n.d.	n.d.	n.d.	n.d.
Total µg fatty acids in 400 µg LPS sample		2.0	Trace	10.0	Trace

Hot phenol water isolated aqueous phase lipopolysaccharides were analysed by GC-MS by the CCRC, Athens, Georgia, USA. Fatty acid composition was performed by FAME derivatisation and consecutive GC-MS analysis. n.d. – not detected. * Mole percent of total carbohydrate.

Analysis of the carbohydrate composition showed that 99.9 - 100% of the total carbohydrates was glucose, depending on the sample (Table 12). The high amount of glucose present was also likely to be responsible for the nonviable total carbohydrate values, most likely over the detection limit of the equipment. Other carbohydrates were also detected: 0.1% Mole Galactose at 15°C (0.1 MPa) and trace under other conditions; trace N-Acetyl Galactosamine (GalNAc) and trace N-Acetyl Glucosamine (GlcNAc) at

15 and 4°C (0.1 MPa); and an unknown Uronic acid under all conditions. Due to the large amount of glucose it was hypothesised that either *P. profundum* SS9R LPS has a glucan component, or that the glucan was a contaminant.

Table 12: Carbohydrate composition of *P. profundum* SS9R LPS grown under different conditions

	Temp/pressure of growth	0.1 MPa 15°C	28 MPa 15°C	0.1 MPa 4°C	28 MPa 4°C
Glycosyl residue (% Mole*)					
<i>Ribose</i>		n.d.	Trace	n.d.	n.d.
<i>Rhamnose</i>		n.d.	n.d.	n.d.	n.d.
<i>Unknown Uronic Acid</i>		Trace	Trace	Trace	Trace
<i>Fucose</i>		n.d.	n.d.	n.d.	n.d.
<i>Xylose</i>		n.d.	n.d.	n.d.	n.d.
<i>Glucuronic Acid</i>		n.d.	n.d.	n.d.	n.d.
<i>Galacturonic Acid</i>		n.d.	n.d.	n.d.	n.d.
<i>Mannose</i>		n.d.	n.d.	n.d.	n.d.
<i>Galactose</i>		0.1	Trace	Trace	Trace
<i>Glucose</i>		99.9	100	100	100
<i>N-Acetyl-Galactosamine</i>		Trace	n.d.	Trace	n.d.
<i>N-Acetyl-Glucosamine</i>		Trace	n.d.	Trace	n.d.
<i>Heptose</i>		n.d.	n.d.	n.d.	n.d.
<i>3 Deoxy-2-manno-2 Octulsonic Acid (KDO)</i>		n.d.	n.d.	n.d.	n.d.
Total µg carbohydrate in 400 µg LPS sample		474.6	695.3	716.0	742.3

Hot phenol water isolated aqueous phase lipopolysaccharides were analysed by GC-MS by the CCRC, Athens, Georgia, USA. Carbohydrate analysis was performed by TMS. n.d. – not detected. * Mole percent of total carbohydrate.

To investigate the linkage of the glucan, the samples were digested with a glucanase enzyme, cellulase, followed by HPLC analysis. Cellulase will only digest 1,4-β-glycosidic linkages, therefore, if glucose was released by the digestion the glucan contained 1,4-β linkages. To calibrate the HPLC a range of concentrations of glucose were analysed and were shown to have a retention time of ~18 minutes (Figure 19A). The anaerobic *P. profundum* SS9R LPS samples from 15°C (0.1 and 28 MPa) and 4°C (0.1 and 28 MPa) were then analysed by HPLC. The LPS samples all appear to have unique profiles by this method (Figure 19B). The samples grown at 0.1 and 28 MPa (15°C) had extra peaks at 7 and 10 minutes that did not appear in the 0.1 and 28 MPa

(4°C) samples. Additionally, the samples taken from 4 and 15°C (0.1 MPa) had peaks at 17 minutes that did not appear in the 4 and 15°C (28 MPa) samples. Since the HPLC showed that each sample had a unique chromatogram, this method may prove to be useful in the future characterisation of LPS composition. Following digestion with cellulase, the LPS samples from all conditions showed a large peak at approximately 18 minutes (Figure 19C). It is likely that the peak observed following cellulase digestion was glucose, as this peak eluted from the column at the same retention time as the glucose peak of the calibration (Figure 19A). To confirm whether the peak at 18 minutes contained glucose, glucose was added to undigested LPS samples and then re-examined by HPLC. The addition of glucose confirmed that the peak at 18 minutes had the same retention time as glucose (Figure 20). This demonstrated that the glucan could be digested and therefore enzymatically removed.

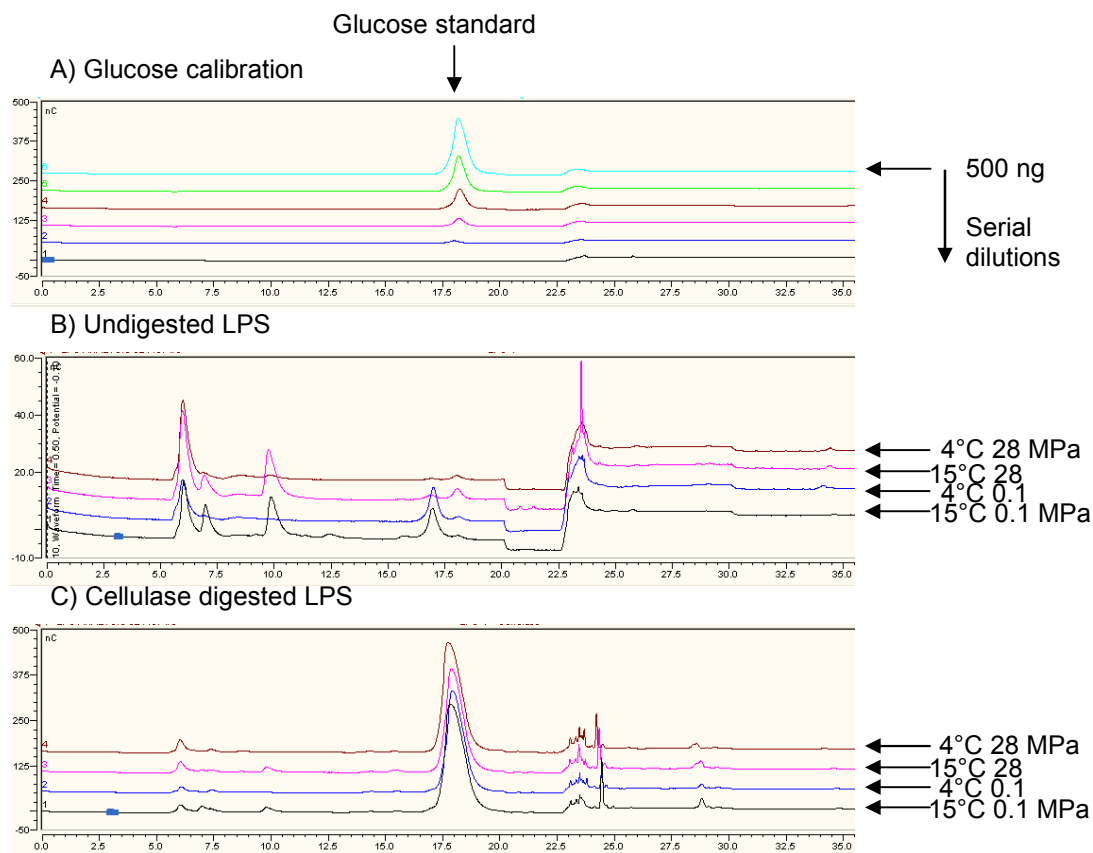


Figure 19: Cellulase digestion of LPS shows that a major component of the LPS may be β 1-4 linked glucose

HPLC High pH Ion exchange chromatography using PA200 column and isocratic gradient of NaOH. Glucose calibration using serial dilutions of glucose suspended in 50 mM sodium acetate pH 5.2 (A). Profile of LPS samples, 1 mg of each sample was resuspended in 50 mM sodium acetate pH 5.2 (B). Profile of LPS samples following 1 hour incubation with 10 units cellulase (*Trichoderma Viride* – Sigma Aldrich) at 37°C (C). Performed in collaboration with Hector Moria.

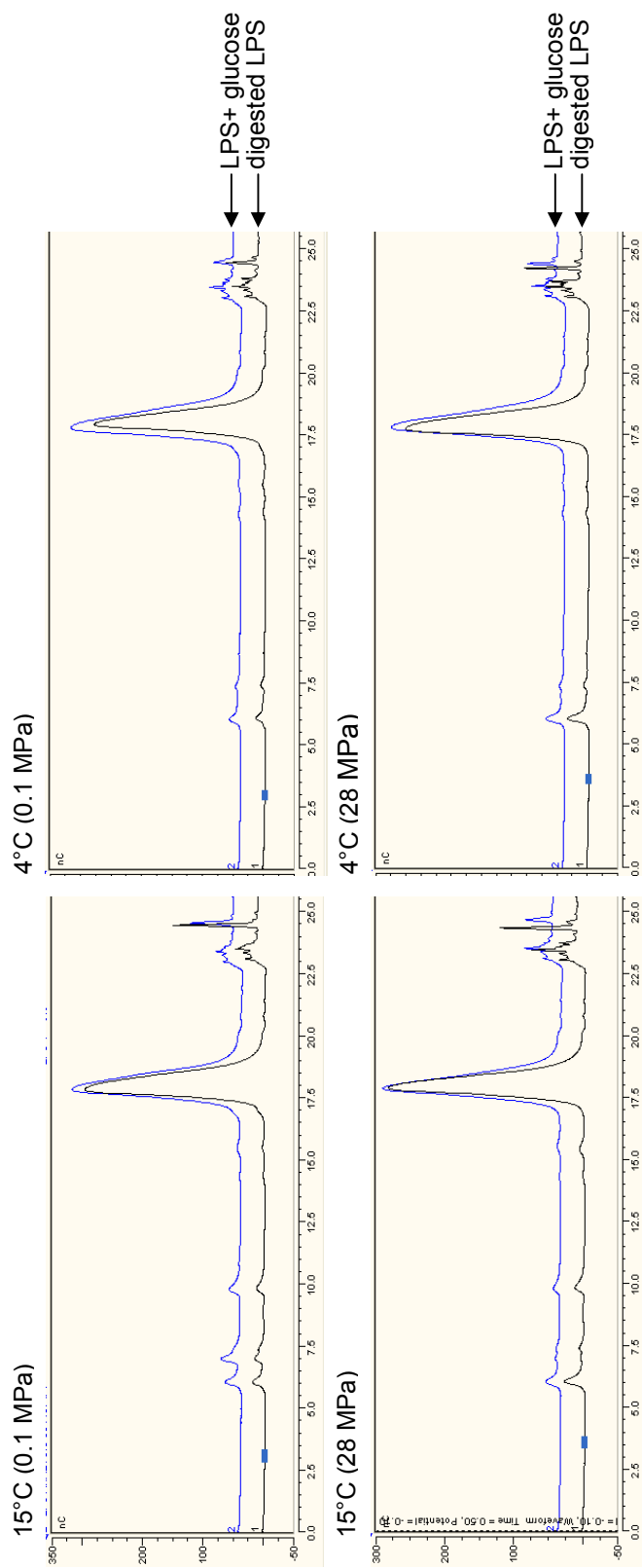


Figure 20: The peak following the cellulase digestion LPS samples most likely contains glucose

HPLC High pH Ion exchange chromatography using PA200 column and isocratic gradient of NaOH. 1 mg of LPS sample was resuspended in 50 mM sodium acetate pH 5.2 and digested for 1 hour incubation with 10 units cellulase (Trichoderma Viride – Sigma Aldrich) at 37°C. Each graph shows undigested LPS with additional 500 ng glucose and cellulase digested LPS. Performed in collaboration with Hector Moria, University of Aberdeen.

To investigate whether the glucan was part of the LPS, the LPS profile of *P. profundum* SS9R grown at 15°C (0.1 MPa) was compared to cellulase digested LPS by DOC-PAGE. If the glucan was detectable, the LPS profile would be expected to change following digestion with cellulase. This analysis showed that the cellulase enzyme itself was detectable by DOC-PAGE as a band at the top of the gel (Figure 21). While the loading of these gels did not represent the same level of detail seen in previous gels, it was sufficient to demonstrate that there was no significant alteration in the LPS following digestion with cellulase. As a control, the glucan cellulose was analysed by this method with and without cellulase digestion. In both cases cellulose did not appear on the gel. A change in the LPS profile was not detected by this method, however this does not rule out the possibility that the glucan is part of the LPS. One possibility is that the glucan does not enter the gel (i.e. it is too large) or that it is a contaminant resulting from the ultracentrifugation process used to purify the freeze-dried LPS.

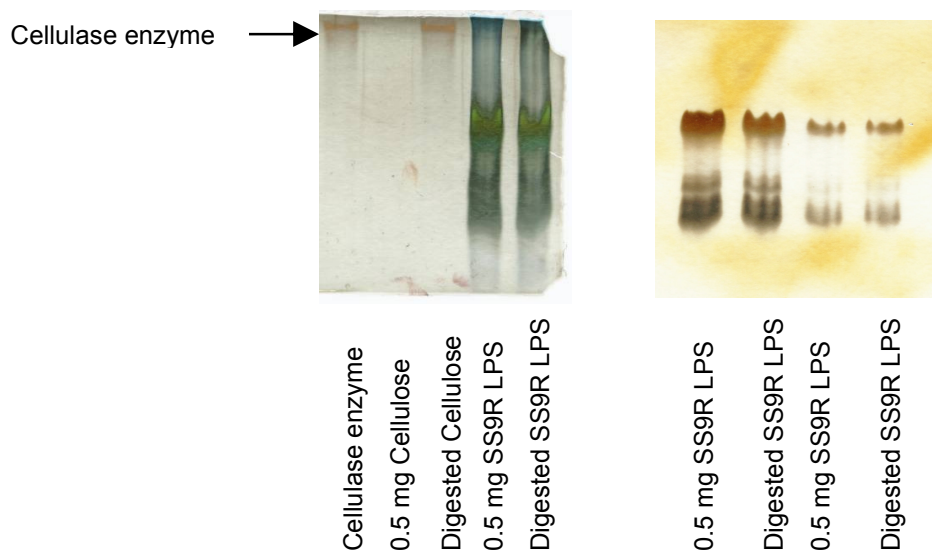


Figure 21: The DOC-PAGE LPS profile of *P. profundum* SS9R was not affected following cellulose digestion

LPS from a *P. profundum* SS9R stationary phase culture, grown at 15°C (0.1 MPa, anaerobic), were isolated by the small scale hot phenol water method. The aqueous phase LPS were then analysed by DOC-PAGE followed by alcian blue silver staining. Each sample was resuspended in 100 µl 50 mM sodium acetate: 10 units cellulase, 0.5 mg cellulose, 0.5 mg cellulose treated with 10 units cellulase for 1 hour at 37°C, 0.5 mg SS9R LPS, 0.5 mg SS9R LPS treated with 10 units cellulase for 1 hour at 37°C. Cellulase enzyme (*Trichoderma Viride* – Sigma Aldrich).

By growing *P. profundum* SS9R aerobically in shaking flasks, it was discovered that the glucose content of the LPS was significantly reduced from 99.9 to 59% (Table 13). Also, the overall amount of carbohydrate present in the sample was reduced from an inaccurate immeasurable amount to just over 22% over the sample. Consequently, the GC-MS composition of these cultures showed a greater number of different carbohydrates than were previously detected from anaerobically grown cultures (Table 12). LPS from aerobically grown cultures contained 59% glucose, compared to 99.9–100% in anaerobically grown cultures. Glucuronic acid and galactose were the next most abundant, with 20.5 and 18% respectively. Xylose and arabinose were found in trace quantities, whereas they were not detected in the previous anaerobic cultures.

Table 13: Carbohydrate composition of *P. profundum* SS9R LPS grown aerobically

Glycosyl residues (% Mole*)	15°C (0.1 MPa) aerobic
Xylose	Trace
Arabinose	Trace
Mannose	1.1
Galactose	18.1
Glucose	58.9
Galacturonic acid	1.4
Glucuronic acid	20.5
Total µg carbohydrate in 500 µg LPS sample	113.9

Hot phenol water isolated aqueous phase lipopolysaccharides were analysed by GC-MS by the CCRC, Athens, Georgia, USA. Carbohydrate analysis was performed by TMS. n.d. – not detected. * Mole percent of total carbohydrate.

Aerobic growth also changed the levels of fatty acids detected, not only in the relative proportions of the fatty acids, but also C16:1 which was now detected in large amounts (Table 14). Fatty acids now comprised 3.6% of the LPS samples, compared to less than 2.5% with anaerobic growth.

Table 14: Fatty acid composition of *P. profundum* SS9R LPS grown aerobically

Fatty Acids (% of total)	15°C (0.1 MPa) aerobic
Dodecanoic Acid (C12:O)	14
Hydroxydodecanoic Acid (C12:OH)	33
Tetradecanoic Acid (C14:O)	11
Hydroxytetradecanoic Acid (C14:OH)	9
Hexadecanoic Acid (C16:O)	7
Hexadecenoic Acid (C16:1)	26
Total µg fatty acids in 500 µg LPS sample	18.0

Hot phenol water isolated aqueous phase lipopolysaccharides were analysed by GC-MS by the CCRC, Athens, Georgia, USA. Fatty acid composition was performed by FAME derivatisation and consecutive GC-MS analysis. n.d. – not detected. * Mole percent of total carbohydrate.

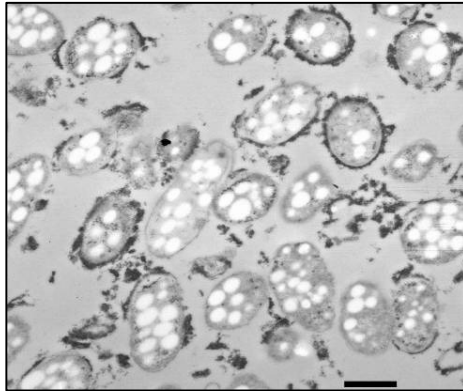
3.4 The effect of temperature and pressure on the expression of Capsular Polysaccharides (CPS)

In addition to LPS and other polysaccharides, the CPS is also regulated in response to environmental conditions. Indeed in *E. coli* K12 and *S. Typhimurium* LT2 CPS expression is dependant upon numerous conditions (Grant *et al.*, 1969). Extracellular polysaccharides have also been shown to be useful for protection against desiccation and freezing damage (Tamaru *et al.*, 2005). The CPS of *P. profundum* SS9R was therefore investigated for any alterations in response to cold temperatures or high pressures.

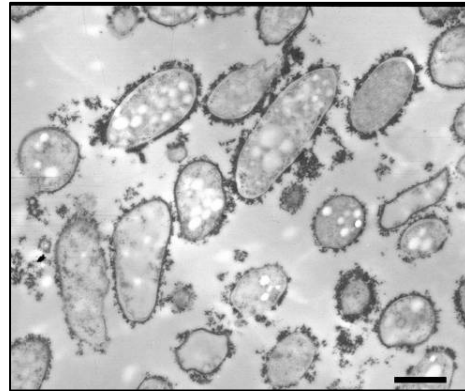
Cationised ferritin is a cationic dye that binds to negatively charged residues and has been used previously to stain the CPS (Nesper *et al.*, 2003). TEM of cationised ferritin stained *P. profundum* SS9R grown under different conditions indicated that among the other microscopic effects (Figure 12) and changes seen in the LPS profiles (Figure 16), the CPS also changed in response to the environment (Figure 22). At 0.1 and 28 MPa (15°C) there was little cationised ferritin staining. The capsule was considerably thicker at 4°C (0.1 MPa) than at 15°C (0.1 MPa).

In all TEM of *P. profundum* SS9R conducted vacuoles were present, which also appear to be significantly enlarged at 28 MPa compared to 0.1 MPa. The vacuoles may be another mechanism for equalising internal pressure, however considerable more work needs to be performed to investigate this feature and to rule out the possibility that this is an artefact of the TEM procedure.

A) 15°C 28 MPa



B) 15°C 0.1 MPa



C) 4°C 0.1 MPa

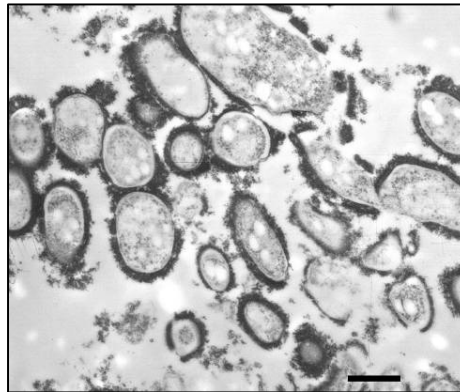


Figure 22: The amount of high molecular weight capsule increases with a reduction in temperature and pressure

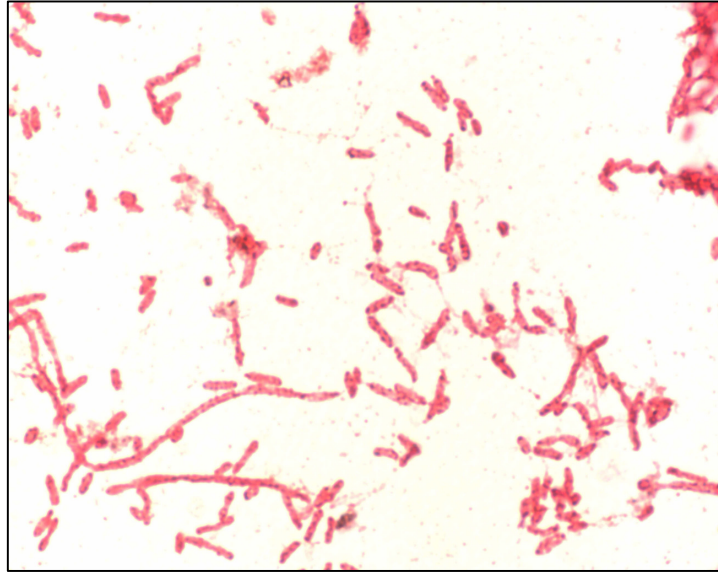
P. profundum SS9R stationary phase cultures from different pressures and temperatures were prepared for TEM analysis with the addition of cationised ferritin (method 2.8.4). 15°C 28 MPa (A), 15°C 0.1 MPa (B) and 4°C 0.1 MPa (C). Bar equals 1µm.

3.4.1 Investigating the composition of the vacuoles

Since *P. profundum* SS9 has been shown to accumulate beta-hydroxybutyrate (β -HB, the basic unit of PHB) (Martin *et al.*, 2002), the vacuoles were hypothesised to be an accumulation of β -HB. PHB is a lipid polymer which has been shown to be used by microorganisms as an osmolyte and as an energy storage molecule (Uchino *et al.*, 2007). The inclusion or exclusion of osmolytes from the cell helps to

maintains fluid balance (Yancey *et al.*, 1982). By staining with Sudan black B and Fuchsin it was possible to assay the accumulation of lipids in *P. profundum* SS9R at different pressures (Burdon *et al.*, 1942). *P. profundum* SS9R appeared to accumulate more lipid granules at 0.1 MPa than at 28 MPa (Figure 23). This finding indicates that the accumulation of lipids was unrelated to the vacuoles seen by TEM (Figure 22). The exact function of the vacuoles and the accumulation of lipids are therefore still unknown.

A) 0.1 MPa (15°C)



B) 28 MPa (15°C)

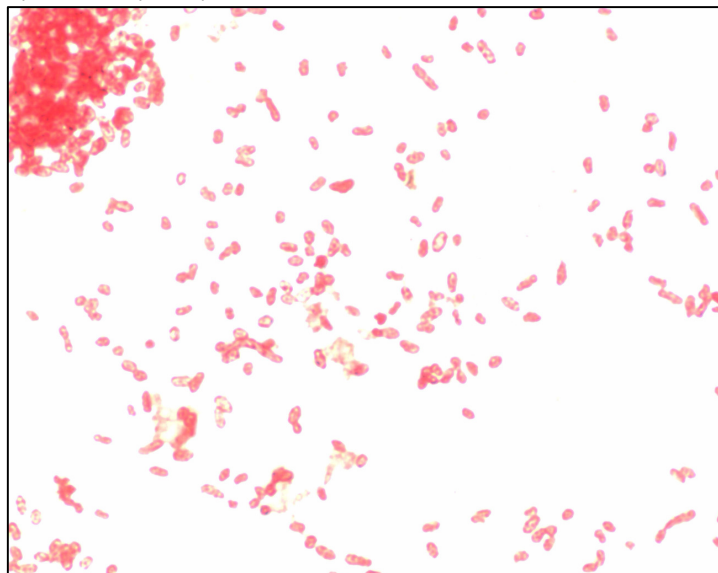


Figure 23: *P. profundum* SS9R accumulates more lipids at atmospheric pressure

Stationary phase cultures of *P. profundum* SS9R from 0.1 MPa 15°C (0.1 MPa, anaerobic) was diluted in fresh marine broth, sealed in plastic Pasteur pipettes and incubated at either 0.1 MPa (15°C, anaerobic) or 28 MPa (15°C, anaerobic). Stationary phase cultures were heat-fixed onto a clean glass slide before being stained with sudan black B, immersed in Xylene and counterstained with Fuchsin. Slides were visualised using 100x oil immersion. Lipid granules appear as dark spots, cells are stained pink. A) 0.1 MPa (15°C), B) 28 MPa (15°C).

3.5 Discussion

3.5.1 *P. profundum* SS9R growth in liquid media was measured by OD₆₀₀

The growth of *P. profundum* SS9R at 15 and 4°C (0.1 MPa) was assessed by both OD₆₀₀ and cfu. It was found that the cfu data from 15 and 4°C (0.1 MPa) was significantly variable, having multiple large peaks and troughs that did not correlate with the OD₆₀₀ data. The variability in the cfu data was greater from 4°C growth than from 15°C growth. This variability may be a result of the experimental procedure, whereby culture from 15 and 4°C (0.1 MPa), were serially diluted, spotted onto marine agar plates and incubated at 15°C (0.1 MPa) to calculate the cfu. It is possible that the transfer from 4 to 15°C in such a manner was stressful to *P. profundum* SS9R, resulting in the variability, an alternative may be to recover the cells at 4°C thereby removing the possible stress of the temperature transfer. However, at the time there was no other suitable alternative method for counting cfu with *P. profundum* SS9R. Measuring OD₆₀₀ has since been demonstrated to be a reliable measure of the growth, and 2 cultures with the same OD₆₀₀ have been shown to have approximately equal numbers of cells. One point to consider was that 0.1 MPa is not the optimal growth condition and it may be necessary to recover for cfu measurements at 28 MPa (15°C). Due to the technical limitations of the growth system, recovery at 28 MPa was not yet feasible. There are however several other methods used for determining bacterial growth. One of which is dry cell weight where the culture is pelleted, dried and weighed. There are also various methods for determining bacterial viability, such as using a viability stain.

3.5.2 *P. profundum* SS9R is a rod-shaped bacterium under optimal growth conditions

P. profundum SS9R grew optimally at 28 MPa (15°C), with a greater exponential growth rate and higher final OD₆₀₀ than at 0.1 MPa (15°C). At 28 MPa 15°C

P. profundum SS9R was visualised by light microscopy to be between 2 – 3 μm in length. At 0.1 MPa (15°C) the cells became filamentous, having increased lengths and in some cases, a pleomorphic morphology. In non-piezophilic bacteria, filamentation has been shown to be a result of elevated pressure, by either damage to or stalling of DNA replication (Gottesman *et al.*, 1981; Yayanos and Pollard, 1969; Zobell and Cobet, 1962). Since *P. profundum* SS9 is a piezophilic bacterium, the replication machinery may have evolved to function optimally at 28 MPa and therefore a reduced pressure may give the same phenotype due to an inability to complete DNA replication or a much reduced replication rate, giving rise to the filaments. This may have consequences on the measurement of viability by cfu. It is important to note that the relationship between OD₆₀₀ and cfu for rod-shaped cells and filamentous cells may be more complex, since the number of bacteria that spawn from a single filamentous cell is unknown and depends on the basis for mechanism of the filamentation.

P. profundum SS9R also exhibited a reduced growth rate at 4°C (0.1 MPa) compared to 15°C (0.1 MPa). While *P. profundum* SS9 is characterised as a psychrophilic bacterium, the optimum growth temperature has been shown to be 15°C (DeLong, 1986). At temperatures either side of 15°C the cellular machinery encounters obstacles, whether they are the same obstacles as at high pressures, such as dissociation of ribosomes and RNA polymerase or the denaturation of essential proteins are questions that are still waiting to be answered.

3.5.3 Growth of *P. profundum* SS9R on marine agar

The growth of *P. profundum* SS9R on a marine agar was also investigated because previous work had shown that *E. coli* cells immobilised to a solid surface had a different protein expression to cold shock than those in liquid media (Frédérique Perrot, 2001). While the growth at 4°C (0.1 MPa) took longer than growth at 15°C (0.1 MPa), both cultures had significant growth at 10⁻⁴ dilution. This was similar to the growth in marine broth, where both cultures reached comparable final OD₆₀₀. If the response to temperature was dependant on growth on solid or liquid media, then the response to

pressure may also differ since cold temperatures and high pressures can exert similar physical effects on bacterial membranes, reducing membrane fluidity (Bartlett *et al.*, 1995; Macdonald, 1984). The growth of *P. profundum* SS9R on marine agar at pressure was therefore also investigated. While this was technically difficult, a previous publication described a method of growing bacteria at high pressure by overlaying the bacteria with agar (Masui and Kato, 1999). Attempts to reproduce this method were initially met with difficulties and the methodology had to be adapted for use with *P. profundum* SS9R. *P. profundum* SS9R is a psychophilic bacterium and the use of molten agar was detrimental to growth. Therefore, a low temperature gelling agar (LTGMA) was purchased which allowed the agar to be poured at a cooler temperature than possible with BactoAgar. The LTGMA was then able to be cooled to 50°C before pouring and with immediate cooling after pouring the detrimental effect of the hot LTGMA was reduced. The overlay of LTGMA reduced the growth of *P. profundum* SS9R at 0.1 MPa (15°C) by at least 100 fold. However, when incubated at 28 MPa (15°C) the lowest dilution of growth was increased to 10^{-4} . This method therefore demonstrated the piezophilic growth of *P. profundum* SS9R on agar, whereby a greater recovery was achieved at 28 MPa compared to 0.1 MPa.

3.5.4 *P. profundum* SS9R LPS alters in response to temperature and pressure

The DOC-PAGE LPS profile of *P. profundum* SS9R altered when grown under different temperatures and pressures. The aqueous phase LPS profiles changed with respect to temperature at 0.1 MPa and with respect to pressure at 15°C. LPS alterations in response to cold temperatures have been documented by GC-MS and NMR in many bacteria including *E. coli* and the psychophilic bacterium *P. syringae* (Carty *et al.*, 1999; Kumar *et al.*, 2002). However, an LPS alteration in response to cold temperatures, such as that seen with *P. profundum* SS9, has not been demonstrated before by DOC-PAGE. Therefore, the non-pressure-adapted close relative of *P. profundum* SS9R, *P. profundum* 3TCK, and the well characterised bacterium

S. Typhimurium LT2 were analysed. Neither bacterium showed an alteration in the LPS profile in response to cold temperatures or high pressures. Consequently, the DOC-PAGE LPS alterations in response to pressure and cold temperatures seen in *P. profundum* SS9 are, at the moment, unique.

3.5.5 GC-MS analysis of *P. profundum* SS9R LPS revealed a glucan

Initial investigation of the polysaccharide composition of *P. profundum* SS9R found that it was comprised of 99.9 – 100% glucose (depending on the sample), most likely in the form of a glucan polymer. The LPS is purified from other cellular material during the hot water phenol preparation and from the CPS and EPS during the ultracentrifugation steps. Therefore the glucan is likely to be part of the LPS and not a contaminant. To resolve that matter the LPS could be radio-labelled to ensure its purity or the outer membrane could be separated from other constituents using sucrose gradients. When taken together with the DOC-PAGE profiles, the GC-MS data may give some insight into the LPS alterations. At 15 and 4°C (28 MPa) the LPS profile by DOC-PAGE appear almost identical. The carbohydrate composition for these conditions are also virtually identical, with the exception of trace ribose present at 15°C (28 MPa). The carbohydrate compositions for the LPS taken from 15 and 4°C (0.1 MPa) are also identical to each other. The LPS samples from 0.1 MPa, however, contain trace N-acetyl-galactosamine and N-acetyl-glucosamine that were absent at 28 MPa. The differences detected in the fatty acid composition could also be argued to mirror the aqueous phase LPS DOC-PAGE profile with stark differences between cultures grown at 0.1 and 28 MPa.

The detection of fatty acids was reduced somewhat and it was hypothesised that the large amount of glucan was interfering with the detection of the fatty acids by GC-MS. It was therefore deemed necessary to identify the glucan in order to remove it and to allow the further analysis of the LPS by GC-MS. By digesting the LPS with cellulase and analysing by HPLC, it was shown that a large amount of glucose was released. Since cellulase is only capable of digesting glucans with a 1,4- β linkage, it

could be assumed that the glucan contains this linkage. However, commercial enzymes may not be 100% pure and therefore the linkage may still be unresolved. The desired result was still reached though, the digestion of the glucan.

It was necessary to investigate whether the cellulase digestion affected the LPS profile and therefore whether the glucan was present in the DOC-PAGE LPS profile, and indeed part of the LPS. Following digestion with cellulase, the LPS from of *P. profundum* SS9R did not change significantly, which indicated that the glucan either did not enter the polyacrylamide gel or that it is not detectable by this method. The exact nature of the glucan therefore remains unknown.

The composition of the LPS was also analysed from aerobically grown cultures. Interestingly, this reduced the overall carbohydrate and glucose content of the LPS samples allowing detection of a significant amount of other carbohydrates and more fatty acids. As previously mentioned, growth conditions can greatly affect the fatty acid and LPS composition of bacteria. Also, because of the shaking culture and length of incubation, rifampicin was also present during aerobic growth. These factors influence the physiology of the bacteria. Nevertheless, this allowed greater detection of the LPS composition by GC-MS. Future GC-MS analysis of *P. profundum* SS9R LPS will therefore be performed on aerobic cultures. The growth of *P. profundum* SS9R under anaerobic and aerobic conditions was not found to be significantly different.

3.5.6 TEM analysis of *P. profundum* SS9R showed the capsule is affected by pressure

Using TEM, it was found that the level of CPS staining by cationised ferritin altered under temperature, specifically having the lowest amount at 0.1 and 28 MPa (15°C) and the greatest amount at 0.1 MPa (4°C). EPS have been shown to have a protective effect against desiccation and freezing damage (Ophir and Gutnick, 1994; Tamaru *et al.*, 2005) and have a supporting role for extracellular enzymes (Marx *et al.*, 2007). The CPS could therefore be acting as an insulating layer to protect against cold temperatures and may help to stabilise the envelope at low pressures and temperatures.

However, in order to further investigate the effects of the capsule we need to examine the cell under elevated temperatures and pressures to determine whether the same effects are seen. In Chapter 5 a CPS deficient mutant, FL9, is investigated to illuminate the effects the capsule has on cold temperature adaptation.

3.5.7 Analysis of *P. profundum* SS9R vacuoles/granules

The TEM also showed the presence of vacuoles in *P. profundum* SS9R, which were most intense at 28 MPa (15°C). The intensity of the vacuoles may be an artefact from TEM processing. Gas vacuoles have been observed in a psychrophilic bacterium (Auman *et al.*, 2006) and in an obligately piezophilic bacteria following decompression (Chastain and Yayanos, 1991). Gas vacuoles are thought to aid the equalisation of cytoplasmic pressure and also motility through the water column (Walsby, 1972). Alternatively, they may not be gas vacuoles, it is also possible that these are aggregations of glycogen or PHB. *P. profundum* SS9 has previously been shown to accumulate several osmolytes and piezolytes in response to elevated pressure and cold temperatures, such as β -HB, the basic unit of PHB (Martin *et al.*, 2002). By staining with Sudan black B and Fuchsin, *P. profundum* SS9R appeared to have accumulated more lipid granules at 0.1 MPa than at 28 MPa, which is the opposite of that seen with the vacuoles by TEM. However, this staining method will stain all lipids and consequently the composition of the granules seen by this method cannot be fully identified. Also, the distribution of the lipid granules did not match the distribution of the vacuoles by TEM and thus the two seem to be unrelated.

3.5.8 Summary

The LPS profile of *P. profundum* SS9R was shown to be altered when the temperature or pressure changed, as visualised by DOC-PAGE. Many bacteria have been shown to alter the composition of the outer membrane in response to environmental stresses, mostly by altering the fatty acid and protein content. Alterations, specifically in

the LPS, have also been documented in many bacteria however this is the first documented case of LPS adaptation in a piezophilic bacterium. This is most likely an adaptive process to maintain the fluidity and integrity of the outer membrane. The model of homeoviscous adaptation explains that membrane content is altered to maintain fluidity. This is usually accomplished by the inclusion of saturated fatty acids, longer chain fatty acids and branched fatty acids.

The precise modifications to the *P. profundum* SS9R LPS were not identified due to the presence of the glucan. Since cold temperature modifications to LPS in other bacteria, such as an altered lipid A in *E. coli* and increased levels of hydroxylated fatty acids in *P. syringae* are not detectable by DOC-PAGE it is likely that the modifications in *P. profundum* SS9R are not the same.

4. Results – Characterisation of the growth and LPS of a putative *P. profundum* SS9 O-antigen ligase mutant, FL26

4.1 Introduction – O-antigen ligase

A random transposon mutagenesis was performed on *P. profundum* SS9 and the resulting mutants were then screened for an increased sensitivity to cold temperatures and high pressures (Lauro *et al.*, 2008). An initial cold temperature sensitivity screen was based on the mutants having a reduced growth at 4°C (0.1 MPa) compared to the parent strain on marine agar plates after 120 hours (Lauro *et al.*, 2008). Cold sensitive mutants obtained were rescreened for cold temperature sensitivity in liquid media by determining the cold sensitivity ratio, i.e. the growth rate at 4°C (0.1 MPa) divided by growth rate at 15°C (0.1 MPa) (Lauro *et al.*, 2008). The mutant FL26 contained a mini-Tn5 transposon insertion in *pbpra0218*, a gene which encodes a putative O-antigen ligase (Lauro *et al.*, 2008) (Figure 24 and Table 15). While the primary sequence homology of the putative O-antigen ligase is low it is important to note that *E. coli*, *Klebsiella pneumoniae* and *P. aeruginosa* O-antigen ligases have all been shown to have low sequence homology but were identified by hydropathy plot due to highly conserved secondary structure (Abeyrathne *et al.*, 2005). Preliminary characterisation of FL26 found that it only exhibited cold temperature sensitivity on marine agar plates and not in marine broth (Lauro *et al.*, 2008).

O-antigen ligase is responsible for the addition of the O-antigen polymer to the rough lipopolysaccharide (LPS) precursor. This is thought to take place on the periplasmic face on the inner membrane (McGrath and Osborn, 1991b; Mulford and Osborn, 1983) although the exact mechanisms are still poorly understood. Bacteria with mutations in genes encoding O-antigen ligases share a number of phenotypes. The most characteristic is a loss of smooth LPS. The absence of smooth LPS can be observed by SDS-PAGE analysis as the loss of the uppermost bands in the profile (Figure 25).

This chapter describes the characterisation of a putative O-antigen ligase mutant of *P. profundum* SS9R, FL26, and the phenotypes that arose from the transposon insertion. Growth under cold temperatures and high pressures, and the structure and expression of LPS were investigated.

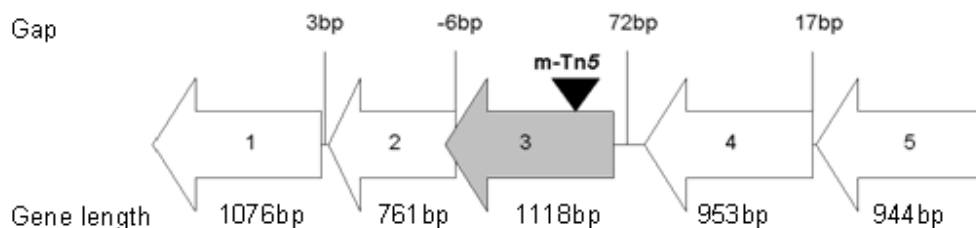


Figure 24: Graphical representation of gene cluster. FL26 contains a mini-Tn5 transposon insertion in *pbpra0218* (Gene 3), which encodes a putative O-antigen ligase
The proteins encoded by the above genes are described in Table 15, along with relative identity and similarity to previously described proteins.

Table 15: Genome annotation and protein homology in *P. profundum* SS9 Chr 1: 223217-228155

	Genome Annotation	Proposed Function	Closest Biochemically Proven Homolog
1	<i>pbpra0216</i>	Glycosyltransferase	71% identity <i>Shewanella putrefaciens</i> 200
2	<i>pbpra0217</i>	Lob1 Glycosyltransferase	40% identity <i>Haemophilus somnus</i> 129PT
3	<i>pbpra0218</i>	O-Antigen Ligase	27% identity <i>Vibrio fischeri</i> ES114
4	<i>pbpra0219</i>	Lipid IVA Acyl Transferase	55% identity <i>Vibrio parahaemolyticus</i>
5	<i>pbpra0220</i>	ADP-L-Glycero-D-mannoheptose 6 Epimerase	82% identity <i>Vibrio fischeri</i> ES114

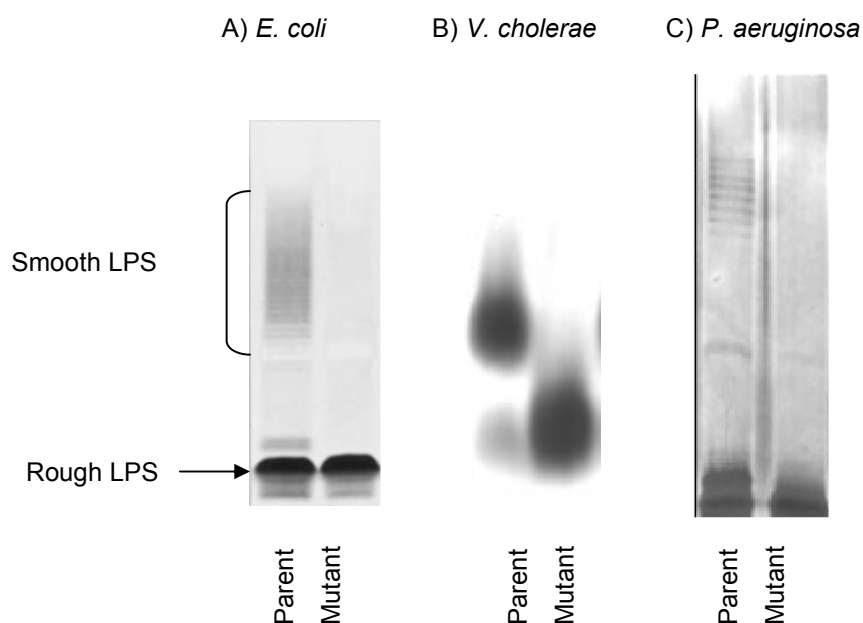


Figure 25: Mutations in O-antigen ligase result in a loss of upper banding pattern by SDS-PAGE

The images show LPS analysed by SDS-PAGE and silver staining. A) *E. coli* F362 pWQ3 (parent), CWG302 pWQ3 (O-antigen ligase mutant) (Heinrichs *et al.*, 1998a). B) *V. cholerae* 01 (parent), 01waaL (O-antigen ligase mutant) (Schild *et al.*, 2005). C) *P. aeruginosa* 01 (parent), PA01 (O-antigen ligase mutant) (Abeyrathne *et al.*, 2005).

4.2 Investigating the cold temperature sensitivity of FL26 relative to the parent strain, SS9R

FL26 was isolated from a screen for cold temperature sensitive mutants based on reduced growth on marine agar plates at 4°C compared to the parent strain (Lauro *et al.*, 2008). This section describes the effects of this mutation on the physiology of FL26; specifically the cold temperature sensitive phenotype on marine agar, in liquid broth and at high pressures.

4.2.1 FL26 displays a cold temperature sensitive phenotype on marine agar

The cold temperature sensitive phenotype of FL26 was initially described as growing “poorly compared to the wild type after 120 hours of incubation at 4°C” on marine agar plates (Lauro *et al.*, 2008). In order to more quantitatively measure the cold temperature sensitive phenotype of FL26, the growth on marine agar was first investigated. Stationary phase *P. profundum* SS9 cultures were pre-grown in marine broth with glucose at 15°C (0.1 MPa). These were diluted to OD₆₀₀ 0.2. Serial dilutions were prepared and spotted in triplicate onto marine agar plates. The plates were incubated at 15, 8, 4 and 2°C (0.1 MPa). At 15°C (0.1 MPa), FL26 showed similar growth to the parent strain, reaching a dilution of 10⁻⁴ (Figure 26). At 8, 4 and 2°C (0.1 MPa) FL26 showed a ten fold reduction in growth, reaching a dilution of 10⁻³, despite increased incubation time (Figure 26). These experiments show that FL26 displays cold temperature sensitive growth on marine agar plates at 8, 4 and 2°C (0.1 MPa) compared to the parent strain that was not seen at 15°C (0.1 MPa). Since the growth of FL26 was identical to *P. profundum* SS9R at 15°C (0.1 MPa), this indicates that FL26 had a cold temperature sensitive phenotype rather than having a general growth defect.

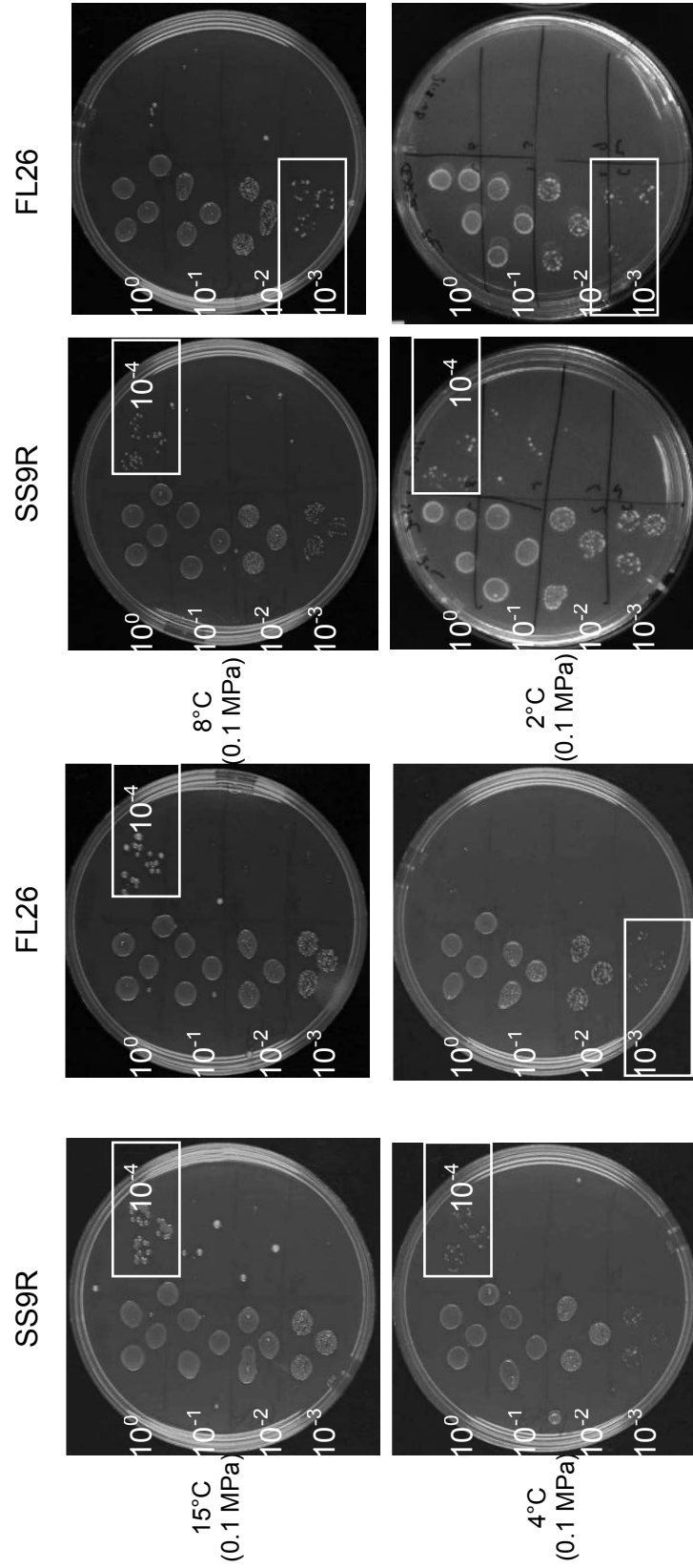


Figure 26: FL26 displays a temperature sensitive growth defect on marine agar at temperatures lower than 15°C

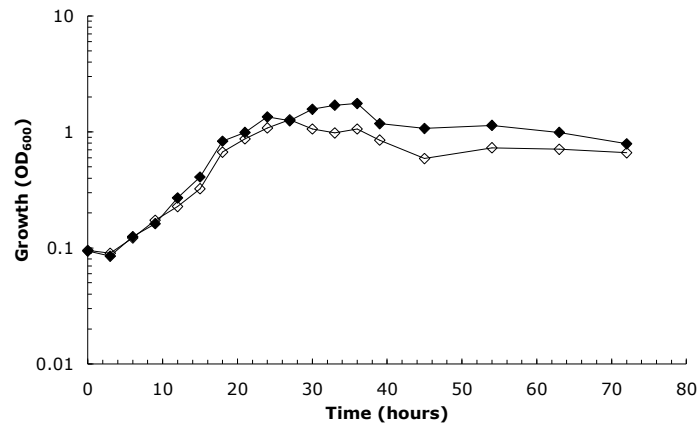
Anaerobic stationary phase cultures of *P. profundum* SS9R and FL26 from 15°C (0.1 MPa) were diluted to OD_{600} 0.2 in fresh marine broth and serially diluted to 10^{-6} . These dilutions were spotted in triplicates of 10 μ l each onto marine agar plates containing 20 mM glucose. The plates were incubated at 15°C, 8°C and 4°C (0.1 MPa) for 10 days at 2°C (0.1 MPa) for 25 days. White boxes highlight the lowest dilution with significant growth.

4.2.2 FL26 does not display a cold temperature sensitive phenotype in marine broth

As described in Section 4.1, cold temperature sensitive mutants were selected for based on a reduced growth on marine agar at 4°C (0.1 MPa) compared to the parent strain (Lauro *et al.*, 2008). After this initial screen, cold temperature sensitive mutants were also assayed for a cold temperature sensitive phenotype in marine broth (Lauro *et al.*, 2008). While the majority of the mutants were also shown to have a cold temperature sensitive phenotype in marine broth, FL26 did not (Lauro *et al.*, 2008). This paper describes the cold sensitivity ratio as the low temperature growth rate divided by that at 15°C (0.1 MPa) (Lauro *et al.*, 2008). In order to more fully describe the growth of FL26 in marine broth, stationary phase cultures that were pre-grown in marine broth with glucose at 15°C (0.1 MPa) were diluted to OD₆₀₀ 0.1 and incubated at 15 and 4°C (0.1 MPa). The OD₆₀₀ was used as the preferred method for measuring growth in liquid media, as described in Chapter 3.

At 15°C (0.1 MPa), FL26 had very similar growth to the parent strain during the early stages of the growth curve (Figure 27A). Upon entry to stationary phase after 25 hours, FL26 had a lower OD₆₀₀ than the parent strain. The growth curve up until late stationary phase was however found to be reproducible. This appeared to be a common feature in *P. profundum* SS9R mutant growth curves, where log phase growth curves were reproducible and the growth in stationary phase was more variable. At 4°C (0.1 MPa) the growth rate was reduced compared to 15°C (0.1 MPa) and FL26 again showed very similar growth to the parent strain over the growth curve (Figure 27B). This indicated that FL26 did not display any significant alteration in growth in marine broth compared to the parent strain. Therefore the cold temperature sensitive phenotype was only detectable on solid marine agar (Figure 26).

A) 15°C (0.1 MPa)



B) 4°C (0.1 MPa)

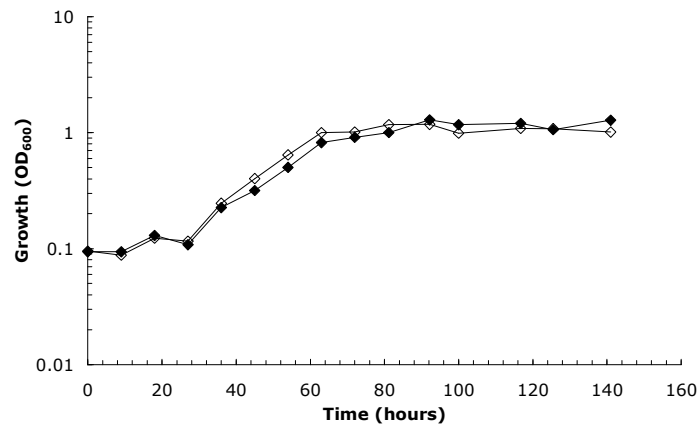


Figure 27: *P. profundum* SS9R and FL26 grow similarly in marine broth at 15°C and 4°C (0.1 MPa)

Anaerobic stationary phase cultures from 15°C (0.1 MPa) were diluted to a starting OD₆₀₀ 0.1 in fresh marine broth with 20 mM glucose and heat sealed in sterile plastic pouches (anaerobic). These were then incubated at the defined conditions. *P. profundum* SS9R (♦) and FL26 (◇). The experiment was performed in duplicate. The above graphs are representative growth curves.

4.2.3 FL26 does not show a high pressure growth alteration using marine agar overlay plates

Until this point the growth of *P. profundum* SS9 at pressure was solely performed using marine broth heat sealed in sterile plastic bags or plastic pasteur pipettes (Allen *et al.*, 1999; Lauro *et al.*, 2008). Since FL26 only displayed a cold temperature sensitive phenotype on marine agar, we reasoned that pressure experiments should also be performed using marine agar. The method developed in Chapter 3.2.3 was utilised to assess the pressure-adapted growth of FL26. Cultures were diluted to an OD₆₀₀ 0.8, serially diluted and spotted in triplicate onto LTGMA. The plates were incubated for 2 hours at 15°C (0.1 MPa) and then overlaid with molten LTGMA.

Non-overlaid LTGMA plates were used to show that FL26 had similar numbers of bacteria before the overlay procedure, with significant growth at 10⁻⁴ dilution (incubated at 0.1 MPa 15°C) (Figure 28). It was shown that overlaid plates for both FL26 and the parent strain had significant growth at 10⁻³ dilution after 6 days incubation at 0.1 MPa (15°C) (Figure 28). After incubation at 28 MPa (15°C), FL26 reached a dilution of 10⁻⁴ (Figure 28). FL26 therefore showed increased growth at 28 MPa compared to 0.1 MPa (15°C), and more importantly, FL26 did not display a high pressure sensitive phenotype compared to the parent strain. Although the overlay method reduced *P. profundum* SS9 growth, increased growth was still noticeable at 28 MPa (15°C). In the future it would be interesting to use this assay to investigate the high pressure adaptation of *P. profundum* SS9 mutant strains at higher pressures and cold temperatures.

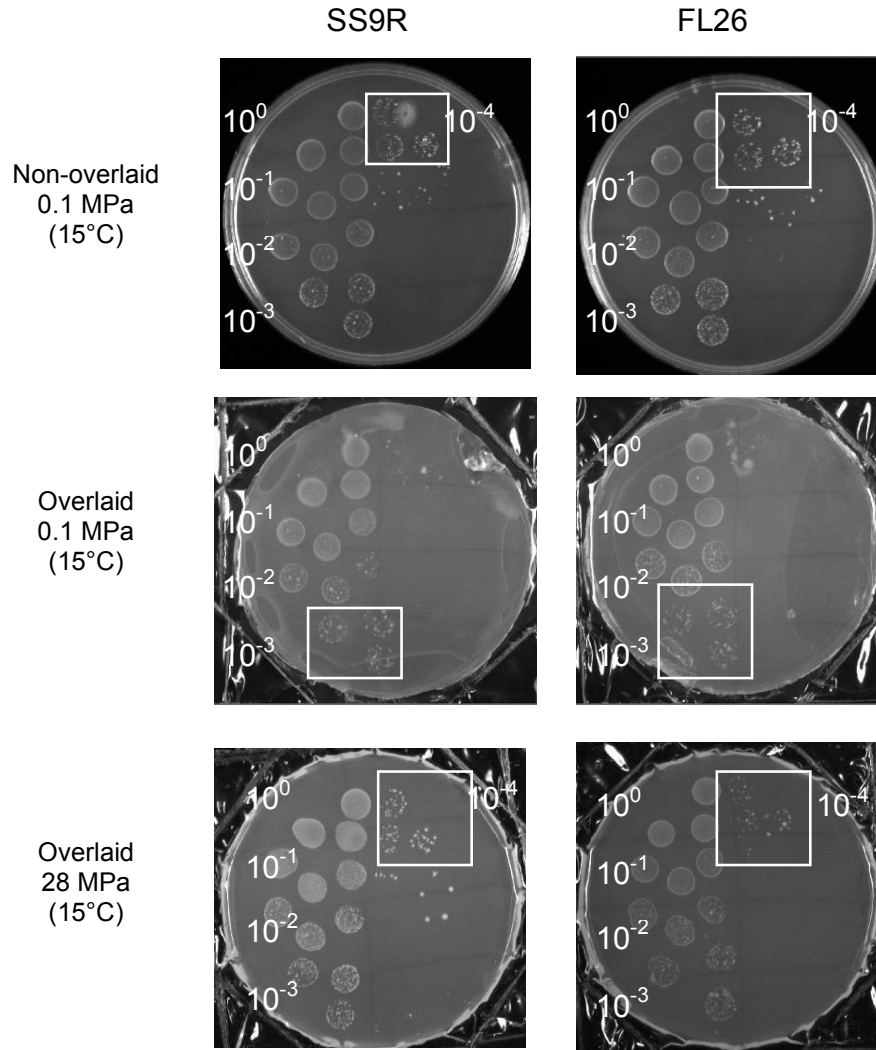


Figure 28: FL26 does not display a high pressure sensitive growth defect on marine agar
 Anaerobic stationary phase cultures of *P. profundum* SS9R and FL26 from 15°C (0.1 MPa) were diluted to an OD_{600} 0.8 in fresh marine broth and serially diluted to 10^{-6} . These dilutions were spotted in triplicates of 10 μ l each onto low temperature gelling marine agar (LTGMA) plates containing 20 mM glucose. The plates were incubated at 0.1 MPa 15°C for 2 hours before being overlaid with molten LTGMA and sealing in sterile plastic bags. The plates were then incubated at 0.1 and 28 MPa (15°C). After 6 days the growth was assessed. White boxes highlight the lowest dilution with significant growth in all 3 spots.

4.3 Analysis of FL26 LPS

FL26 contains a mini-Tn5 transposon insertion in *pbpra0218*, a gene which encodes a protein with significant homology to O-antigen ligase (Figure 24 and Table 15). O-antigen ligase has been demonstrated to be required for the addition of O-antigen to the rough-LPS precursor, which takes place on the periplasmic face of the inner membrane (McGrath and Osborn, 1991b; Mulford and Osborn, 1983). It has been shown that O-antigen ligase utilises ATP for the ligation (Abeyrathne and Lam, 2007), despite a lack of understanding of the actual mechanism of ligation. Since the general effects of O-antigen ligase mutations are well established (i.e. a major alteration in the LPS profile by SDS-PAGE (Figure 25)), this section demonstrates that *pbpra0218* does in fact encode an O-antigen ligase. This was achieved by comparing the LPS from FL26 to that from the parent strain.

4.3.1 The LPS profile of FL26 shows alterations that are characteristic of an O-antigen ligase mutant

The LPS profile of *P. profundum* SS9R was demonstrated by isolation of the LPS using the SDS-lysis method and separation by SDS-PAGE followed by alcian blue silver staining (Chapter 3, Figure 15B). Alcian blue is a cationic dye that has been widely used to stain for acidic LPS such as bacterial capsular LPS (Powell *et al.*, 1982) and has been used to stain isolated LPS in polyacrylamide gels (Corzo *et al.*, 1991). The LPS profile of FL26 had several major differences compared to the parent strain. FL26 lacked the major upper band which is believed to relate to the smooth LPS (the LPS molecule and full length O-antigen) (Figure 29). The intermediate bands that can be clearly seen in the parent strain are also missing in FL26 (Figure 29). These alterations appear similar to those published for other O-antigen ligase mutants in *S. Typhimurium*, *E. coli*, *P. aeruginosa* and *V. cholerae* (Abeyrathne *et al.*, 2005; Heinrichs *et al.*, 1998a; Schild *et al.*, 2005), however the *P. profundum* SS9 strains maintain a number of faint upper bands that O-antigen ligase mutant LPS comparisons do not (labelled “highest

bands”). Whether these relate to a higher molecular weight LPS band, an aggregation of LPS molecules or something else is currently unknown. There are methodologies for the purification of bands from PAGE and subsequent analysis GC-MS, using such a method could provide insights into the nature of the polysaccharide alterations.

Due to the fact that the FL26 cold temperature sensitivity phenotype was observed only on solid marine agar, the LPS from growth on marine agar and in marine broth were compared. At both 15°C (0.1 MPa) and 4°C (0.1 MPa) the parent and FL26 showed differences in the LPS profile when grown on marine agar compared to being grown in marine broth. The parent strain showed alterations in the relative intensities of the intermediate bands when grown on solid agar compared to marine broth, while FL26 appeared to have an extra band immediately above the rough LPS when grown on marine agar (Figure 29). These changes in the relative intensities of the intermediate bands of the parent strain LPS profile were different at 15°C compared to 4°C (0.1 MPa). The alterations in the LPS profile of FL26 grown on marine agar appeared not to be temperature dependant, looking very similar at 15°C (0.1 MPa) and 4°C (0.1 MPa) (Figure 29). This suggested that O-antigen ligase may be necessary to perform the LPS modification seen in *P. profundum* SS9R when grown on agar or from temperature changes and consequently that these were changes in the O-antigen component of the LPS.

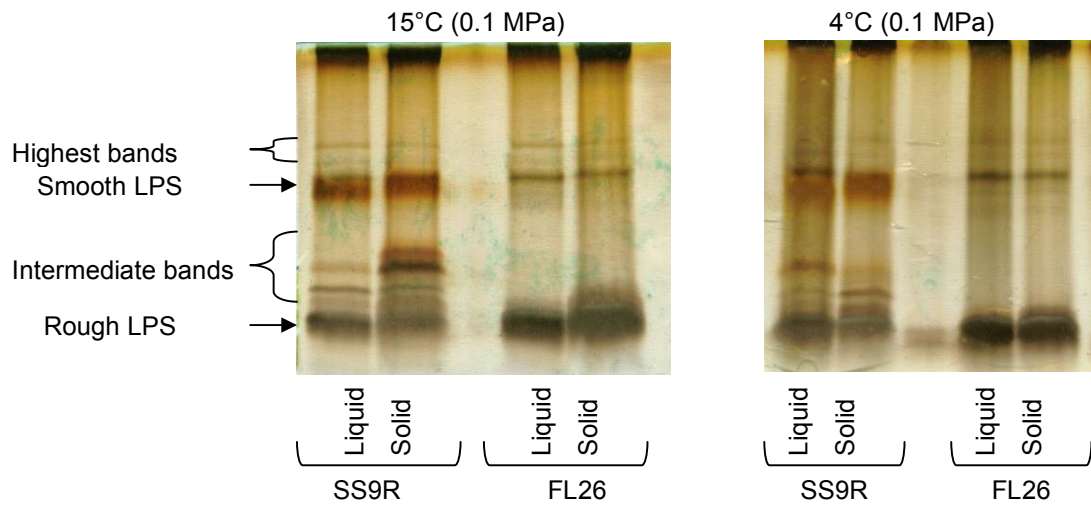


Figure 29: Disruption of the putative O-antigen ligase affects the ability of *P. profundum* to adapt LPS profile in response to temperature and growth on agar

Cultures of *P. profundum* SS9R and FL26 were incubated at 15°C (0.1 MPa) and 4°C (0.1 MPa) in marine broth (anaerobic) and on marine agar plates. Stationary phase cultures from marine broth were pelleted by centrifugation. Colonies grown on marine agar plates were collected and resuspended in fresh marine broth before being pelleted. The LPS were isolated by SDS-lysis and then analysed by SDS-PAGE followed by alcian blue silver staining.

In order to understand more about the effect of the O-antigen ligase mutation in *P. profundum* SS9R on the biochemistry of the LPS, the LPS was also isolated by the hot phenol water method. By using the hot phenol water method and DOC-PAGE the LPS profile can be analysed in greater detail and this also enables the LPS to be purified for biochemical analyses. A litre of culture was grown at 15°C (0.1 MPa aerobic shaking 200 rpm) and used for the extraction. The resulting LPS samples were analysed by DOC-PAGE (Figure 30) and GC-MS (Table 16 and Table 17).

The DOC-PAGE of the aqueous phase LPS showed the same general phenotype as the SDS-PAGE (Figure 29) with FL26 showing a loss of the smooth LPS, but still retaining the rough LPS (Figure 30). The majority of the LPS was isolated in the aqueous phase, with only the rough LPS being found in the phenol phase. This was expected, as LPS lacking the O-antigen are more hydrophobic and can be isolated in the phenol phase. Smooth LPS is expected to be hydrophilic and is therefore only isolated into the aqueous phase. For this reason, the GC-MS analyses were performed on the aqueous phase LPS only.

GC-MS analysis was used to measure the composition with regard to carbohydrates and fatty acids. Since in Chapter 3.3.4, the compositional analysis depended greatly on the growth of the culture, this analysis was performed on aerobically grown cultures rather than anaerobically grown. This allowed the detection of carbohydrates and fatty acids in significant quantities. Firstly, the carbohydrates were investigated, since FL26 is an O-antigen ligase mutant, differences in carbohydrate composition were expected. FL26 had a significant reduction in mannose and galactose compared to the parent (Table 16). The parent LPS also contained trace amounts of xylose and arabinose that were absent in FL26. Mannose, galactose and arabinose have all been previously demonstrated to make up the O-antigen of various *V. cholerae* serotypes (Chatterjee and Chaudhuri, 2003). This suggests that the *P. profundum* SS9 O-antigen consists of mannose and galactose, possibly with traces of xylose and arabinose.

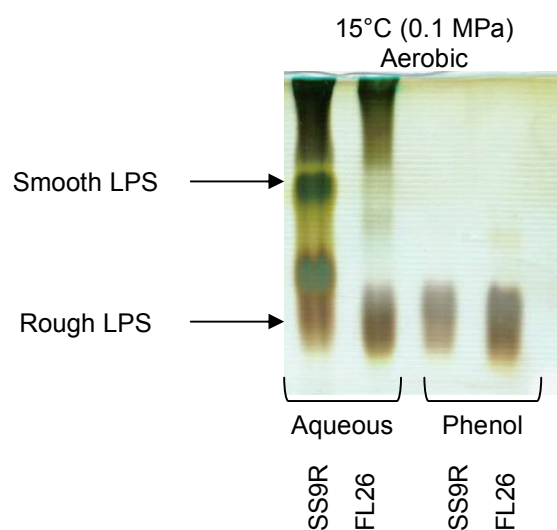


Figure 30: The DOC-PAGE LPS profiles of *P. profundum* SS9R and FL26

Stationary phase cultures from 15°C (0.1 MPa anaerobic) were diluted to a starting OD₆₀₀ 0.1 in 1 litre of fresh marine broth and were incubated at 15°C (0.1 MPa shaking 200 rpm) or aerobically in a plastic bag. Once in stationary phase the cultures were pelleted by centrifugation and the LPS was isolated by the hot phenol water method. The LPS was then visualised by DOC-PAGE followed by alcian blue silver staining.

Table 16: Carbohydrate composition of *P. profundum* SS9R and FL26 LPS by TMS GC-MS

Carbohydrate (% Mole)	SS9R	FL26
Xylose	Trace	n.d.
Arabinose	Trace	n.d.
Mannose	1.1	0.3
Galactose	18.1	10.6
Glucose	58.9	71.1
Galacturonic acid	1.4	1.3
Glucuronic acid	20.5	16.7

Hot phenol water isolated aqueous phase lipopolysaccharides were analysed by GC-MS by the CCRC, Athens, Georgia, USA. Carbohydrate analysis was performed by TMS. n.d. – not detected. * Mole percent of total carbohydrate.

The fatty acid composition was analysed because it was unclear if carbohydrate alterations in the LPS would lead to fatty acid modifications. Also bacteria have been

shown to alter fatty acid content in response to temperature changes. *E. coli* has been thoroughly documented to increase the level of total unsaturated fatty acids in response to low temperatures (Marr and Ingraham, 1962), *P. syringae* incorporates more hydroxylated fatty acids into LPS at lower temperatures (Kumar *et al.*, 2002) and *P. profundum* SS9 alters its total fatty acid composition in response to temperature and pressure with an increase in C18:1 and C20:5 at high pressures (Allen *et al.*, 1999). Therefore, it was possible that the fatty acid content was altered as an adaptation to the loss of O-antigen in the LPS. The fatty acid composition of the LPS was assayed by the Complex Carbohydrate Centre (Table 17).

The fatty acid composition indicated that FL26 had a reduction in the amount of C14:OH and C16:0 compared to the parent strain. FL26 also had some minor increases in C12:0 and C12:OH compared to the parent strain. These alterations could be indicative of further changes to the FL26 LPS composition as an adaptive process to cope with the loss of O-antigen. Longer chain fatty acids are linked to increased membrane fluidity (Sinensky, 1971), the decrease in the levels of C16:0 and C14:OH, and the reciprocal increase in C12:0 and C12:OH might indicate a less viscous membrane and be a possible explanation for the cold temperature sensitivity.

Table 17: Fatty acid composition of *P. profundum* SS9R and FL26 LPS by FAME GC-MS

Fatty Acid (% of total)	SS9R	FL26
C12:0	14	22
C12:OH	33	39
C14:0	11	11
C14:OH	9	3
C16:0	7	2
C16:1	26	24

Hot phenol water isolated aqueous phase lipopolysaccharides were analysed by GC-MS by the CCRC, Athens, Georgia, USA. Fatty acid composition was performed by FAME derivatisation and consecutive GC-MS analysis. n.d. – not detected. * Mole percent of total carbohydrate.

4.3.2 LPS alterations can affect other processes, such as biofilm formation and motility

Although FL26 was shown to have an LPS alteration relative to the parent strain (Figure 29), it has been shown previously that alterations in LPS can influence other cellular processes. These include biofilm formation in *V. cholerae* (Nesper *et al.*, 2001) and motility in *E. coli* and *P. aeruginosa* (Abeyrathne *et al.*, 2005; Komeda *et al.*, 1977).

The O-antigen ligase mutant, FL26, only displays a cold-temperature sensitive phenotype on marine agar and not in marine broth (Lauro *et al.*, 2008) (Figure 26 and Figure 27). Since the phenotype appeared to relate to attachment to a solid surface, the ability of FL26 to form a biofilm was assessed. Cultures were incubated at 15°C (0.1 MPa aerobic shaking 200 rpm) in glass test tubes and the attachment to tubes was visually inspected. After 24 hours the parent strain had a ring of attachment at the air-liquid interface. This attachment was present at all time points, however after 72 hours the attachment was significantly weaker than at earlier time points (Figure 31).

FL26 showed very little attachment after 24 hours. After 48 hours, more attachment could be seen and at 72 hours the parent and FL26 had very similar levels of attachment (Figure 31). However, at this time point the parent was showing lower attachment compared to earlier time points.

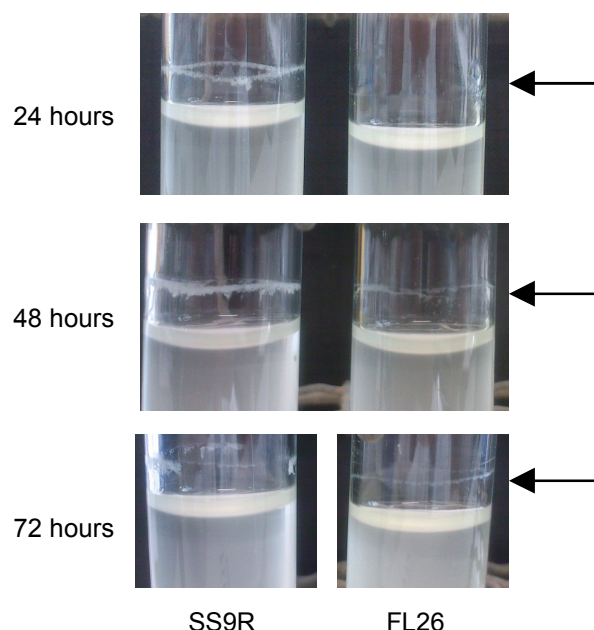


Figure 31: FL26 has reduced biofilm formation compared to *P. profundum* SS9R

Anaerobic stationary phase cultures from 15°C (0.1 MPa) were diluted to an OD₆₀₀ 0.1 in fresh marine broth with 20 mM glucose and incubated at 15°C (0.1 MPa 200 rpm aerobic) for up to 72 hours.

Biofilm formation and motility have been previously shown to be linked in *S. Typhimurium* (Stafford and Hughes, 2007), therefore the motility of FL26 was also investigated to pursue the mechanism behind the alteration in biofilm formation. Also, since mutations in LPS are known to affect motility in *S. Typhimurium* (Toguchi *et al.*, 2000) and *P. aeruginosa* (Abeyrathne *et al.*, 2005), FL26 was compared to the parent strain to investigate whether the O-antigen ligase mutation had an effect on motility. Cultures were spotted onto marine motility plates containing 0.3% (w/v) agar and the diameter of movement was measured daily. After 48 hours of incubation, FL26 had significantly greater motility than the parent strain (Figure 32). From 120 hours onwards, the difference between the two strains appeared consistent.

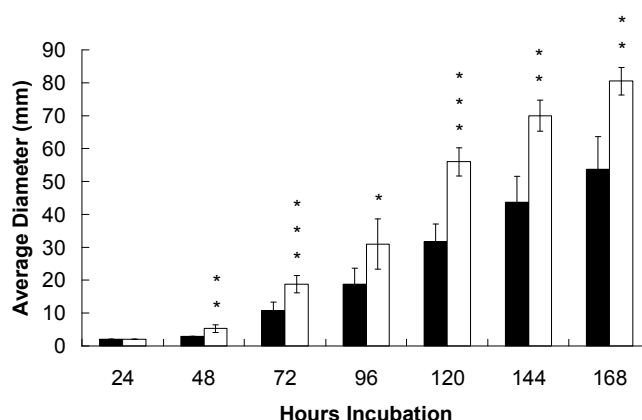


Figure 32: FL26 displays increased motility compared to *P. profundum* SS9R

Anaerobic stationary phase from 15°C (0.1 MPa) cultures were diluted to an OD₆₀₀ 1.0 in fresh marine broth and 3 µl was spotted onto the centre of a motility plate containing 0.3% w/v agar and 20 mM glucose. The plates were then incubated at 15°C and the diameter of measured each day. The average diameter was calculated from 3 motility plates with 2 diameter measurements each. *P. profundum* SS9R (filled), FL26 (white). After 7 days all of the FL26 plates had been completely filled. The mean motility was compared with SS9R using the unpaired student's t-test, 2 tailed: P>0.05 not significant (no symbol), P<0.05 (*), P<0.01 (**), P<0.001 (***). Error bars represent ± 1 SD.

To investigate whether the increased motility was due to an alteration in flagella structure or expression, the flagella were visualised by microscopy. A modified Leifson flagella stain was first used, and while this was demonstrated to work with *V. cholera* (Clark, 1976) and *S. Typhimurium* (Figure 42), the stain did not detect any significant amounts of flagella with *P. profundum* SS9 (data not shown). The flagella were then visualised by TEM, however this also proved inconsistent and most of the visualised flagella were broken or incomplete (data not shown). A second light microscopy method using the fluorescent dye NanoOrange, was successfully performed in collaboration with the Scripps Institute of Oceanography, University of California, USA (Figure 33). This method does not require the bacteria to be processed in any way and therefore successfully showed complete *P. profundum* SS9 flagella. FL26 expressed a flagellum that was comparable to that of the parent strain (Figure 33).

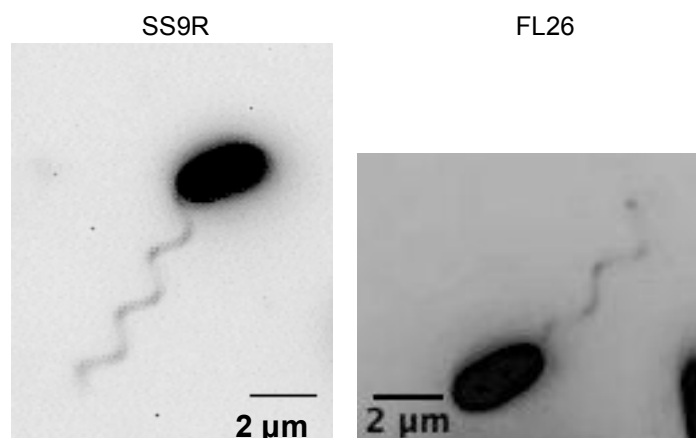


Figure 33: SS9R and FL26 do not have significantly significant flagella

P. profundum SS9R and FL26 were grown on marine agar plates at 15°C (0.1 MPa) and were removed and resuspended in fresh marine broth. A 0.5 μ L aliquot of fluorescent protein stain NanoOrange (Molecular Probes, Invitrogen) was added to 10 μ L bacterial solution and then visualised using an inverted epifluorescence microscope. Bar equals 2 μ m. Performed by E. Eløe, Scripps Institute of Oceanography, University of California, USA.

4.4 Other cold temperature sensitive putative polysaccharide biosynthesis mutants share similar phenotypes to FL26

From the selection of *P. profundum* SS9 cold temperature sensitive transposon insertion mutants, a number of other putative polysaccharide biosynthesis mutants were also identified (Lauro *et al.*, 2008). These mutants have LPS alterations relative to the parent strain that were visualised by SDS-PAGE (Figure 34). The LPS alterations in FL25, FL26 and FL9 were best seen using sodium-m-periodate silver staining (Figure 34A). FL25 had an alteration similar to FL26, where the smooth LPS was not detected. FL9 contains a transposon in a putative tyrosine protein kinase and is discussed in Chapter 5. The LPS alterations in FL3, FL5 and FL7 were better seen with alcian blue silver staining (Figure 34B). FL3, FL5 and FL7 appear to have similar alterations, where they are missing an intermediate band compared to the parent strain. These gels also demonstrate that LPS alterations may require more than one staining

method in order to be characterised. In this case the alcian blue silver staining appears more sensitive to the intermediate bands than the sodium-m-periodate silver staining method (Figure 34B).

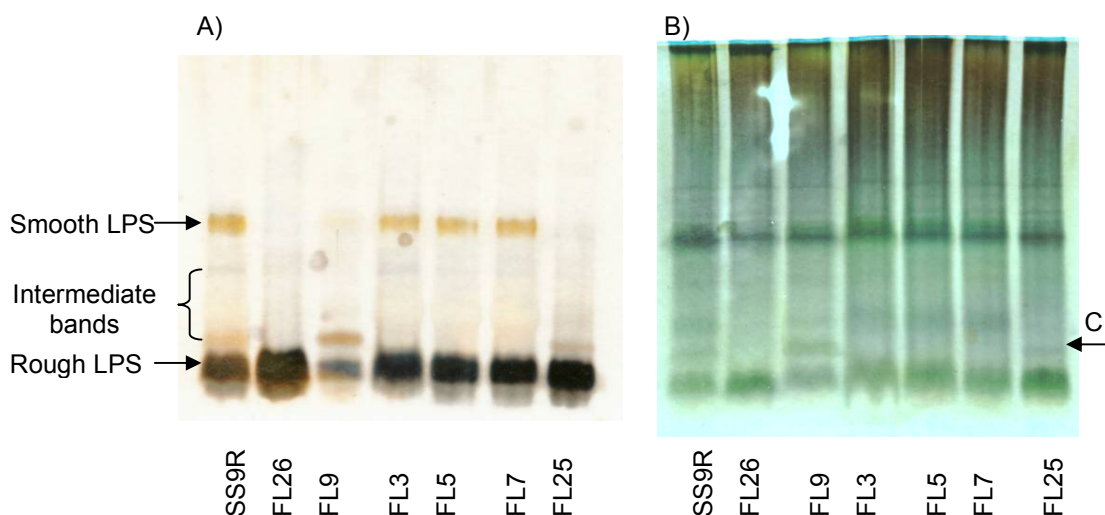


Figure 34: *P. profundum* SS9 transposon mutants in putative LPS biosynthesis genes display alterations in LPS profile by SDS-PAGE

Anaerobic cultures of *P. profundum* were incubated at 15°C (0.1 MPa) in marine broth. *P. profundum* SS9R, FL26 – O-antigen ligase mutant, FL9 – *wzc* mutant, FL3 – glycosyltransferase mutant, FL5 – a second glycosyltransferase mutant, FL7 – *wzx* mutant, and FL25 – *tagF* mutant. Stationary phase cultures from marine broth were pelleted by centrifugation. The LPS were isolated by SDS-lysis and then separated by SDS-PAGE followed by sodium-m-periodate silver staining (A) or alcian blue silver staining (B). Arrow indicates location of intermediate band missing in FL3, FL5 and FL7 (C).

To further investigate whether there was a connection between cold temperature sensitivity and biofilm formation, we also assessed the ability of the cold temperature sensitive polysaccharide mutants to form biofilms. All the mutants showed alterations in the biofilm development relative to the parent strain (Figure 35). The parent strain formed a ring of attachment at the air-liquid interface and the intensity of the ring reduced with prolonged incubation. FL25 was the only mutant tested that appeared to have a ring of attachment similar to the parent strain. FL3 had a significantly increased ring of attachment compared to the parent strain. FL7 and FL5 had a reduced ring of

attachment compared to the parent strain. FL7, FL25 and FL5 also had an area of attachment below the air-liquid interface that was not seen in the parent strain. As with the parent strain, the degree of attachment for all mutant strains reduced after the first 24 hours.

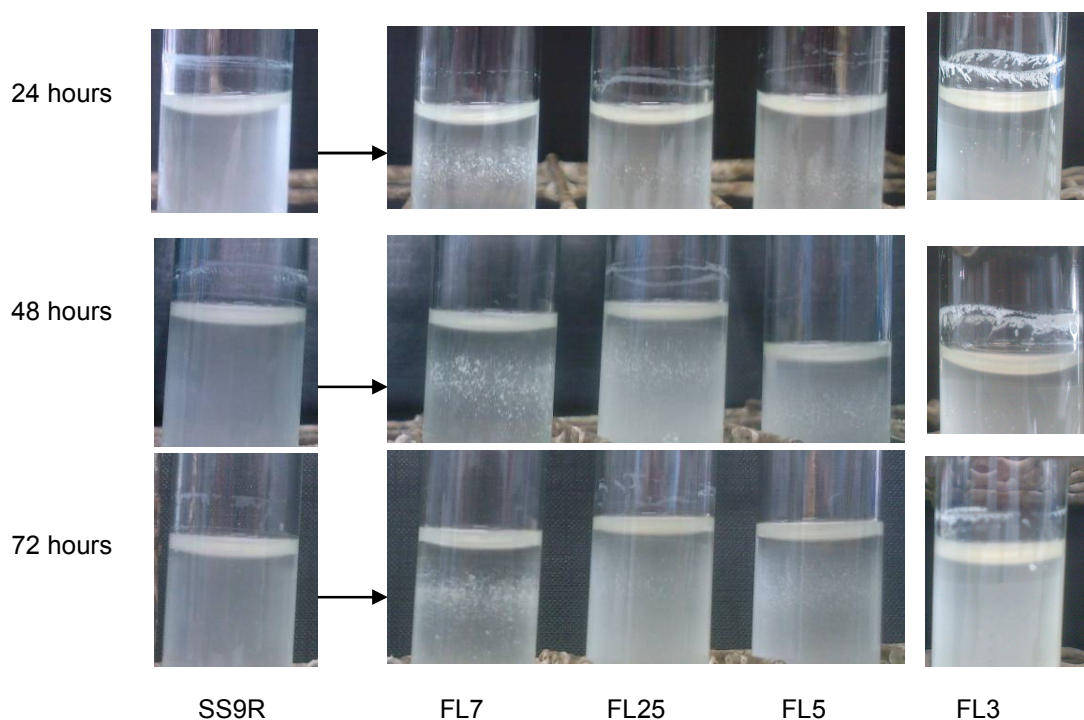


Figure 35: LPS biosynthesis mutants have altered biofilm formation compared to *P. profundum* SS9R

Anaerobic stationary phase cultures from 15°C (0.1 MPa) were diluted to an OD₆₀₀ 0.1 in fresh marine broth with 20 mM glucose and incubated at 15°C (0.1 MPa) 200 rpm (aerobic) for up to 72 hours. *P. profundum* SS9R, FL3 - glycosyltransferase mutant, FL5 - a second glycosyltransferase mutant, FL7 - *wzx* mutant, and FL25 - *tagF* mutant. Arrows indicates the attachment to glass below the air-liquid interface.

The motility of the LPS mutants was then assayed to investigate whether, like FL26, they have both biofilm and motility alterations. The mutants FL3, FL5 and FL7 had significantly greater motility than the parent strain (Figure 36). The movement of FL25 was found not to be significantly different from the parent strain.

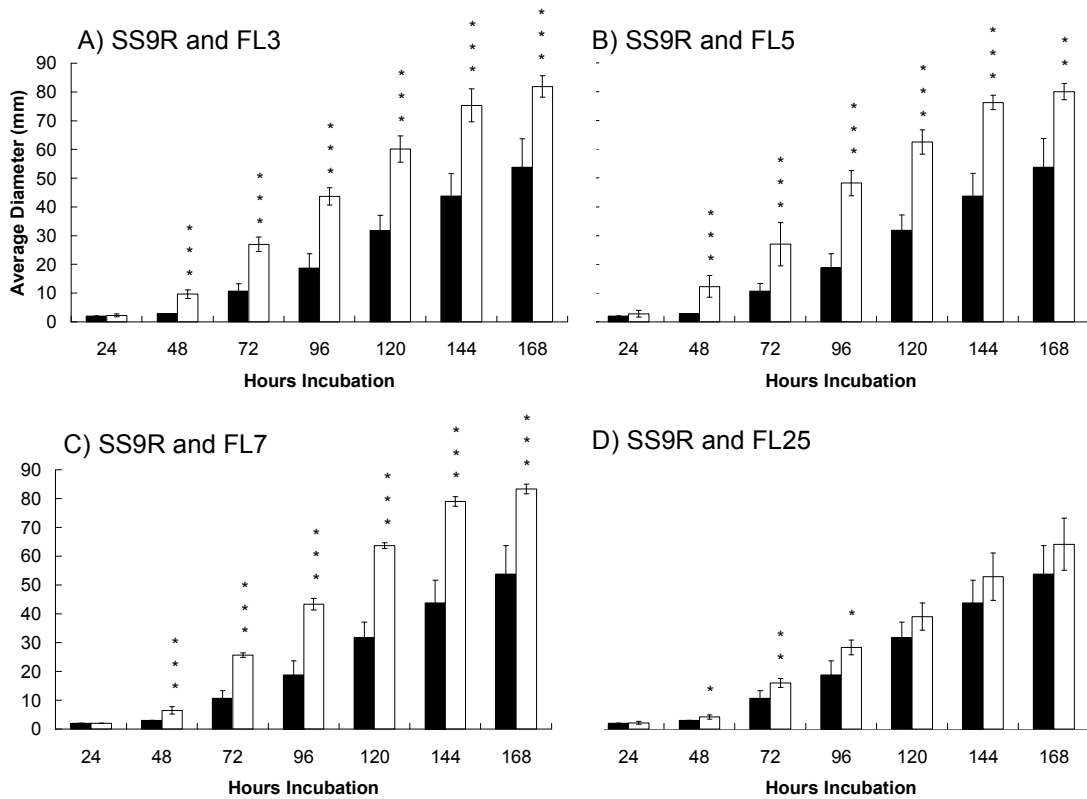


Figure 36: LPS mutants display increased motility compared to *P. profundum* SS9R

Anaerobic stationary phase cultures from 15°C (0.1 MPa) were diluted to an OD₆₀₀ 1.0 in fresh marine broth and 3 µl was spotted onto the centre of a motility plate containing 0.3% w/v agar and 20 mM glucose. The plates were then incubated at 15°C and the diameter of measured each day. The average diameter was calculated from 3 motility plates with 2 diameter measurements each. *P. profundum* SS9R (filled), FL3 - glycosyltransferase mutant (A), FL5 - a second glycosyltransferase mutant (B), FL7 - *wzx* mutant (C), FL25 - *tagF* mutant (D). The mean motility was compared with SS9R using the unpaired student's t-test, 2 tailed: P>0.05 not significant (no symbol), P<0.05 (*), P<0.01 (**), P<0.001 (***). Error bars represent ± 1 SD

Due to the cold temperature sensitive phenotype of the LPS mutants, the motility at 4°C (0.1 MPa) was also investigated. The movement across the plates was slower

compared to at 15°C (0.1 MPa), this was most likely in relation to the reduced growth rate. For example, the parent strain showed no measureable movement until the third week (Figure 37). FL3, FL5 and FL7 still showed significantly increased motility compared to the parent strain, in fact the difference between the movement of the parent strain and FL3 and FL5 at 4°C was significantly greater than at 15°C. However, the movement of FL26 was not significantly different to the parent strain due to the large variation in diameters. FL25 was found to have significantly increased motility compared to the parent strain.

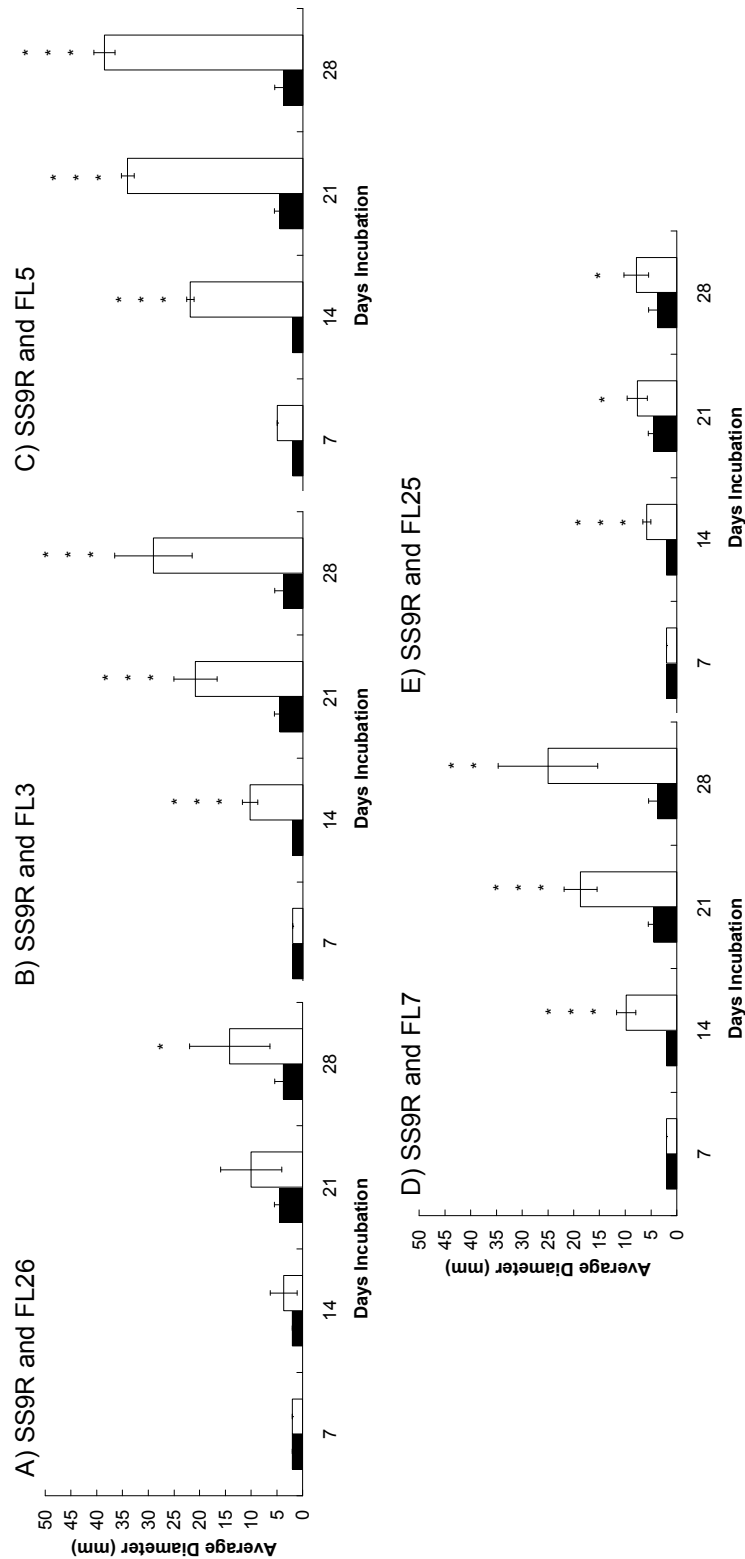


Figure 37: LPS mutants displays increased motility compared to *P. profundum* SS9R at 4°C

Anaerobic stationary phase cultures from 15°C (0.1 MPa) were diluted to an OD₆₀₀ 1.0 in fresh marine broth and 3 µl was spotted onto the centre of a motility plate containing 0.3% w/v agar and 20 mM glucose. The plates were then incubated at 4°C and the diameter of measured each day. The average diameter was calculated from 3 motility plates with 2 diameter measurements each. *P. profundum* SS9R (filled), FL26 (A), FL3 glycosyltransferase mutant (B), FL5 a second glycosyltransferase mutant (C), FL7 wzx mutant (D) and FL25 tagF mutant (E). The mean motility was compared with SS9R using the unpaired student's t-test, 2 tailed: P>0.05 not significant (no symbol), P<0.05 (*), P<0.01 (**), P<0.001 (***). Error bars represent ± 1 SD

4.5 A comparison with the *S. Typhimurium* LT2 O-antigen ligase mutant, SL3749

The biosynthesis of *S. Typhimurium* LPS and the mutant phenotypes which arise from disruptions in those genes have been thoroughly investigated (Ames *et al.*, 1974; Kaniuk *et al.*, 2004; Sirisena and Sanderson, 1994; Toguchi *et al.*, 2000). For this reason, *S. Typhimurium* LT2 and the O-antigen ligase mutant SL3749 were used as a comparison with *P. profundum* SS9R and FL26.

4.5.1 The loss of O-antigen in *S. Typhimurium* LT2 does not lead to cold temperature sensitive growth

The LPS from *S. Typhimurium* LT2 and SL3749 was isolated by SDS-lysis and separated by SDS-PAGE followed by sodium-m-periodate silver staining. The LPS profile demonstrates the characteristic lack of O-antigen and the subsequent loss of the laddering pattern (Figure 38A).

In order to investigate whether the loss of O-antigen would render *S. Typhimurium* LT2 temperature sensitive, the growth on LB agar plates at 15°C was compared to the growth at 37°C, in a similar manner to the *P. profundum* SS9 experiment (Figure 26). Both strains grew comparably at 37°C, with significant growth seen up until the 10⁻⁵ dilution (Figure 38B). At 15°C there was no significant difference between the parent and mutant growth, again growing to 10⁻⁵ dilution (Figure 38). Growth at 15°C took 3 days longer than at 37°C, although this was the same for both the parent and mutant strain. Under these conditions, a loss of the O-antigen in *S. Typhimurium* LT2 did not appear to affect cold temperature sensitivity on LB agar.

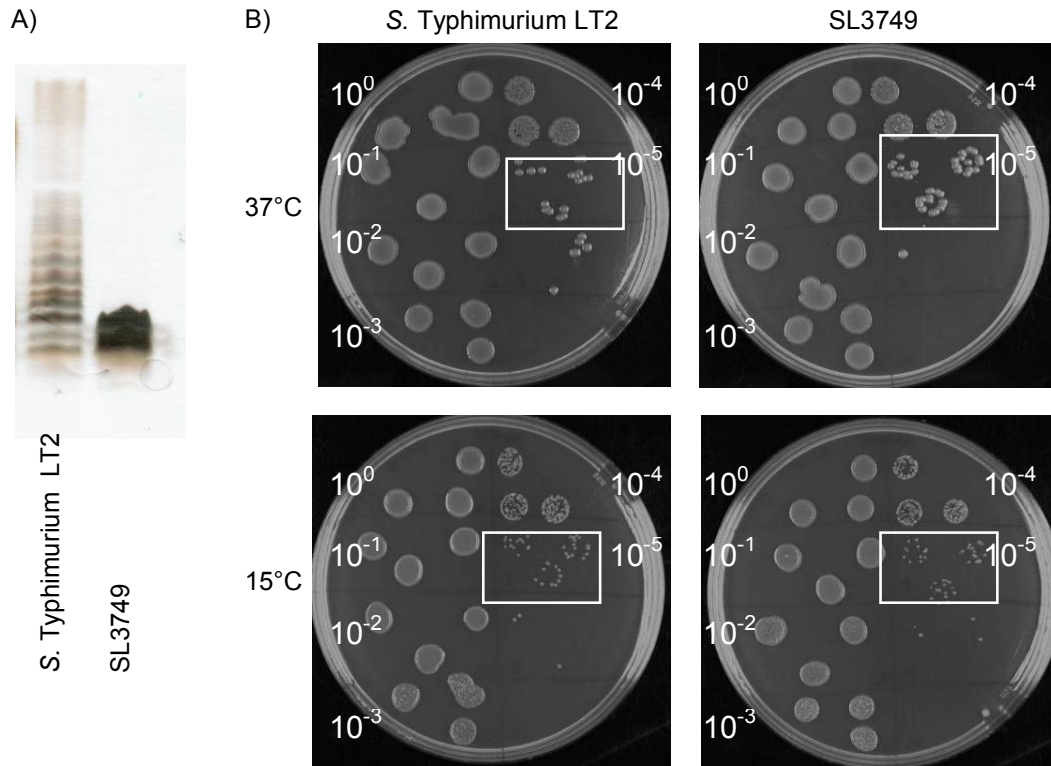


Figure 38: *S. Typhimurium* LT2 and SL3749 do not display a cold temperature sensitive phenotype on LB agar

A) Stationary phase cultures of *S. Typhimurium* LT2 and SL3749 (*waaL*-) from 37°C shaking culture were pelleted by centrifugation. The LPS were isolated by SDS-lysis and then analysed by SDS-PAGE followed by sodium-m-periodate silver staining.

B) Stationary phase cultures of *S. Typhimurium* LT2 and SL3749 (*waaL*-) were diluted to an OD_{600} 0.1 in fresh LB and serially diluted to 10^{-6} . These dilutions were spotted in triplicates of 10 μ l each onto marine agar plates. The plates were incubated at 37°C and 15°C. After 1 (37°C) and 4 days (15°C) the growth was assessed.

The growth of *S. Typhimurium* LT2 and SL3749 at cold temperatures was also investigated in LB broth to assess whether any growth defects existed that were not apparent on LB agar. *S. Typhimurium* LT2 and SL3749 grew similarly at 37°C (Figure 39A). At 15°C, the growth rate was reduced compared to 37°C but both the parent and mutant strain showed similar growth (Figure 39B). SL3749 therefore displayed no significant cold temperature sensitivity at 15°C compared to the parent strain.

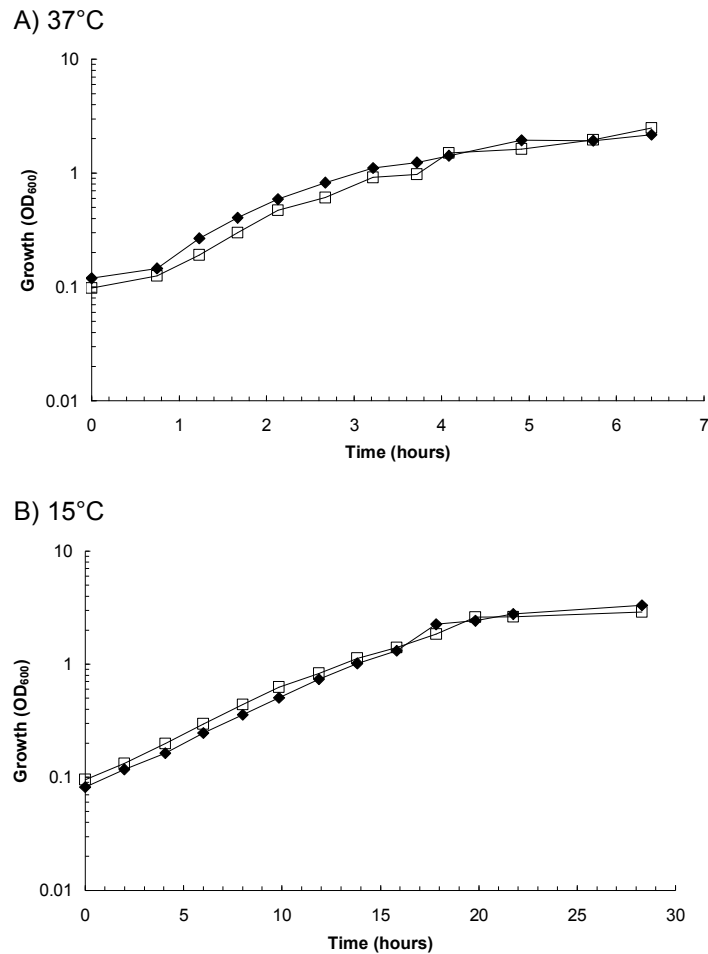


Figure 39: SL3749 does not display a temperature sensitive phenotype in 15°C shaking liquid culture

Stationary phase cultures were diluted to an OD₆₀₀ 0.1 in fresh LB and incubated at 37°C (A) and 15°C (B), 200 rpm. The optical density was measured frequently as a measure of growth. *S. Typhimurium* LT2 (♦) and SL3749 (*waaL*-) (□).

4.5.2 *S. Typhimurium* LT2 does not alter LPS profile in response to cold temperatures or growth on agar

P. profundum SS9R exhibited LPS alterations when grown on solid medium compared to liquid (Figure 29). To investigate whether this alteration also occurred in *S. Typhimurium* LT2, the LPS was analysed from growth at 37°C and 15°C in both LB broth and on LB agar. The LPS profile did not appear to alter significantly when the temperature was reduced or when grown on LB agar compared to LB broth (Figure 40). This may however be because the changes in LPS were too subtle to be detected by SDS-PAGE and that a more sensitive analytical method may be required to detect any such changes.

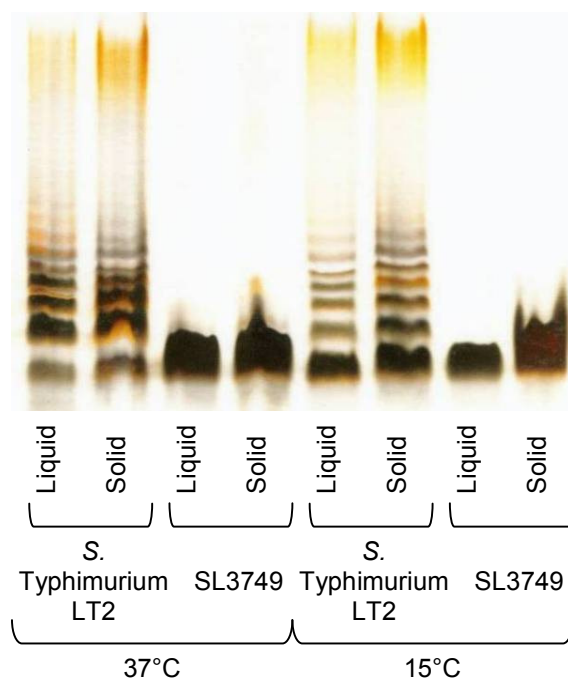


Figure 40: The LPS profile of the *S. Typhimurium* LT2 and SL3749 do not alter when grown on solid media or with reduction in temperature

Stationary phase cultures of *S. Typhimurium* LT2 and SL3749 (*waaL*-) from 37°C and 15°C liquid cultures were pelleted by centrifugation. Colonies grown on LB agar plates at 37°C and 15°C were collected and resuspended in fresh LB before being pelleted. The LPS were isolated by SDS-lysis, separated by SDS-PAGE and visualised by sodium-m-periodate silver staining.

4.5.3 SL3749 has a reduction in swimming motility compared to the parent strain

FL26 was shown to have an increased motility on 0.3% (w/v) marine agar compared to *P. profundum* SS9R (Figure 32). LPS alterations are known to affect motility, for example *P. aeruginosa* O-antigen mutants can no longer move by swimming or swarming motility (Abeyrathne *et al.*, 2005). The motility of SL3749 on 0.3% (w/v) LB agar was assessed and showed significantly less motility than the parent strain. The parent strain moved an average of 50 mm after 7 hours incubation, whereas SL3749 had not yet moved significantly from the point of inoculation (Figure 41). At 12 hours the parent strain had filled all plates (petri dish is 85 mm in diameter) and SL3749 had moved an average diameter of 10 mm. Following overnight incubation SL3749 also filled the motility plates, confirming that *S. Typhimurium* LT2 O-antigen ligase mutants had a reduced motility.

One explanation for the reduction in motility could be an alteration in the structure or expression of the flagella. A modified Leifson flagella stain was used which loads dye onto the flagella allowing it to be visualised by light microscopy (Clark, 1976). This method showed that the parent strain had numerous flagella, with some detached flagella also present in the background (Figure 42). SL3749 also had a large number of flagella, although a lot were detached flagella in the background, more so than with the parent strain. However this is difficult to quantify microscopically. Interestingly the two strains also showed different aggregation phenotypes, with the parent strain forming clumps whilst SL3749 did not. This is most likely due to the smooth and rough LPS phenotypes, respectively.

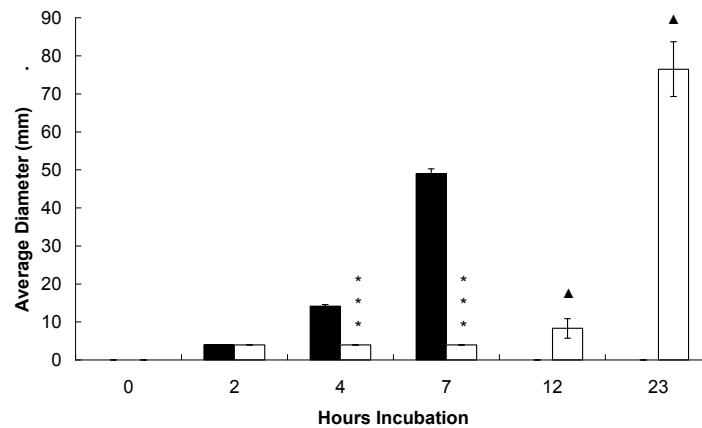


Figure 41: SL3749 displays reduced motility compared to the parent strain

Stationary phase cultures were diluted to an OD_{600} 1.0 in fresh LB and 3 μ l was spotted onto the centre of an LB motility plate containing 0.3% w/v agar and streptomycin. The plates were then incubated at 37°C and the diameter of measured frequently. The average diameter was calculated from 3 motility plates with 2 diameter measurements each. *S. Typhimurium* LT2 (filled) and SL3749 (*waaL*-) (white). After 12 hours all of the *S. Typhimurium* LT2 plates had been completely filled and were no longer plotted. The mean motility was compared with SS9R using the unpaired student's t-test, 2 tailed: $P > 0.05$ not significant (no symbol), $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), untested (▲). Error bars represent ± 1 SD.

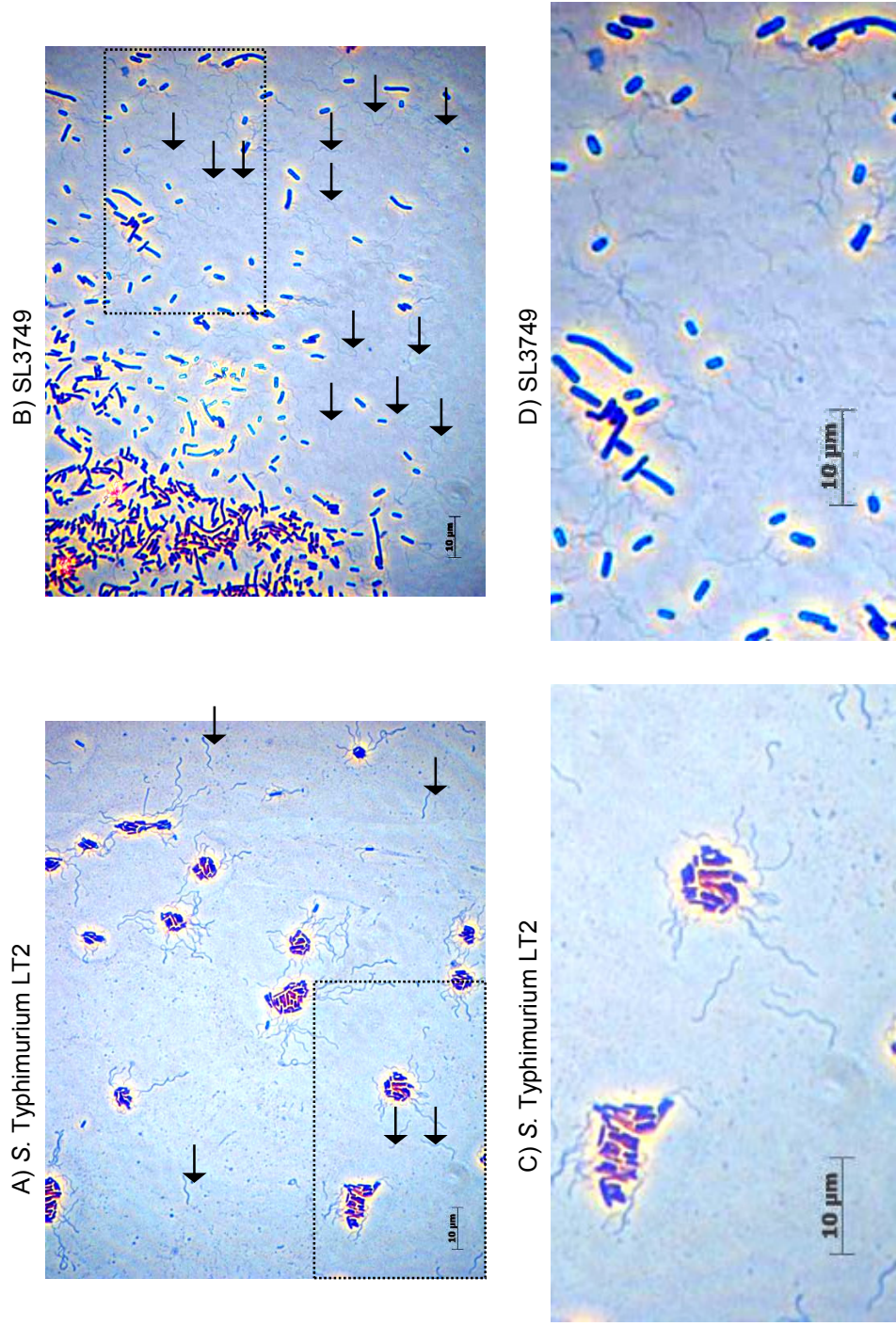


Figure 42: SL3749 has an increase in the amount of detached flagella compared to the parent strain
 Stationary phase cultures and were stained for flagella (Clark, 1976) (see methods 2.8.3). (A) *S. Typhimurium* LT2 and (B) SL3749 (*waaL*⁻). Arrows indicate detached flagella. (C) and (D) are magnified views of the boxed areas in (A) and (B).

4.6 Complementation of *P. profundum* SS9R FL26

4.6.1 The *P. profundum* SS9 complementation vector pFL190

A limiting factor for the complementation of *P. profundum* SS9 mutants was the lack of suitable vectors. One vector that was chosen was pFL190, an arabinose inducible expression vector that was designed for use with *P. profundum* SS9 (Figure 43) (Lauro *et al.*, 2005). The vector has been demonstrated to be capable of driving the expression of a cloned gene in a broad range of hosts, including *P. profundum* SS9 (Lauro *et al.*, 2005). *lacZ* is a gene with numerous uses in cloning. *lacZ* encodes β -Galactosidase which will cleave β -galactosides such as lactose into monosaccharides. When used in conjunction with X-Gal the β -Galactosidase activity will result in a blue colour. *lacZ* has been cloned into pFL190 (pFL191) and shown to express β -Galactosidase in the presence of arabinose in marine broth (Lauro *et al.*, 2005). The regulation of β -Galactosidase by pFL190 on marine agar plates was investigated because the cold temperature sensitive phenotype of FL26 was only visible on marine agar plates.

P. profundum SS9R pFL191 was grown to stationary phase, diluted to a specified OD₆₀₀ and serially diluted across marine agar plates containing X-Gal and a range of arabinose concentrations. Blue colonies indicated the expression of the β -Galactosidase. An X-Gal marine agar plate containing 0.36% (w/v) glucose showed that in the absence of arabinose *P. profundum* SS9R pFL191 colonies remained white (Figure 44G). On plates that contained arabinose *P. profundum* SS9R pFL191 colonies were blue (Figure 44A-E). Also, on a plate containing 0.36% (w/v) glucose and 1% (w/v) arabinose the colonies were also blue, indicating that the expression of β -Galactosidase was not susceptible to catabolite repression (Figure 44F).

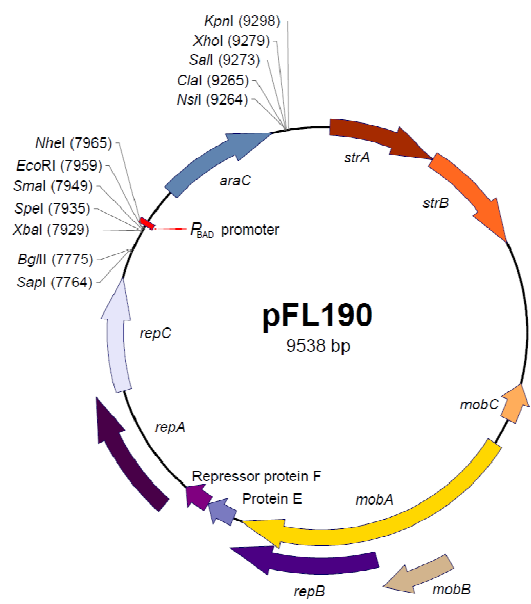


Figure 43: Map of the complementation vector, pFL190

pFL190 was the vector used for complementation of *P. profundum* SS9 mutants. *P_{BAD}* arabinose inducible promoter. *araC* regulator or *P_{BAD}*. *mobA*, *mobB* and *mobC* promote conjugal transfer. *strA* and *strB* streptomycin resistance genes. *repA*, *repB* and *repC* bacterial replication initiators (Lauro *et al.*, 2005)

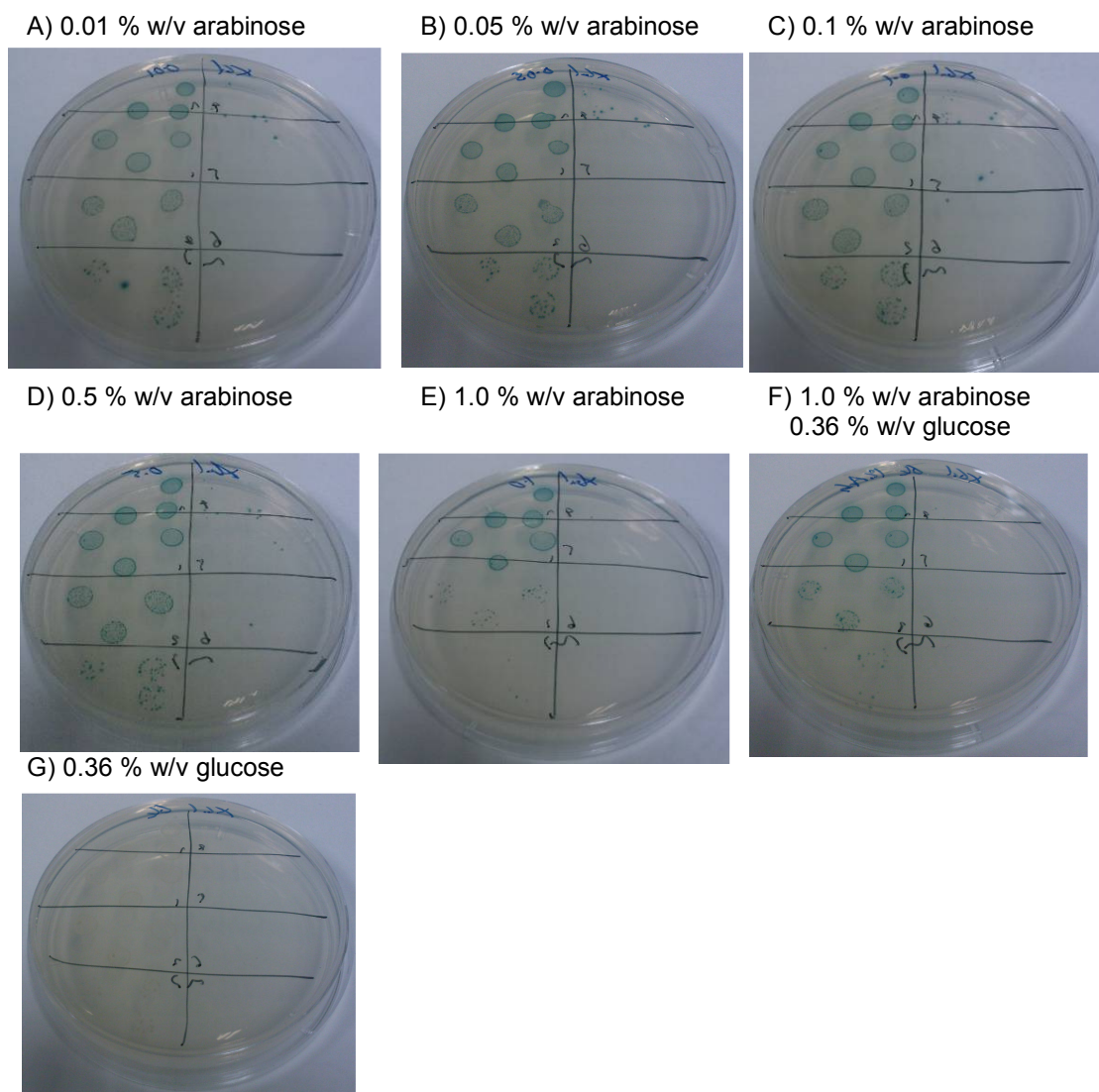


Figure 44: Induction of pFL191 with arabinose produces blue colonies

An anaerobic stationary phase culture of *P. profundum* SS9R pFL191 from 15°C (0.1 MPa) was diluted to an OD_{600} 0.2 in fresh marine broth and serially diluted to 10^{-6} . These dilutions were spotted in triplicates of 10 μ l each onto marine agar, X-Gal, streptomycin plates. The plates were incubated at 15°C (0.1 MPa) for 4 days. (A) 0.01% w/v arabinose, (B) 0.05% w/v arabinose, (C) 0.1% w/v arabinose, (D) 0.5% w/v arabinose, (E) 1.0% w/v arabinose and (F) 1% w/v arabinose and 0.36% w/v glucose and (G) 0.36% w/v glucose.

4.6.3 Expression of pDA01 (pFL190 *pbpra0218*) will partially complement the LPS profile alteration in FL26

To complement the phenotypes seen in FL26, the O-antigen ligase gene (*pbpra0218*) was amplified by PCR, digested with *EcoRI* and *XbaI* and cloned into pFL190 (see Appendix A). This construct was transformed into *E. coli* DH5 α and screened by restriction digest. The successful clone was sequenced, to confirm that the gene sequence was correct and that the promoter region on pFL190 was also intact. This new construct, now pDA01, was then mated into FL26 using the *E. coli* helper strain CSH56 with pRK2013. The ability of pDA01 to complement the cold temperature sensitive phenotype was then tested. At 15°C the parent and FL26 had similar growth on all plates, with and without arabinose. The addition of streptomycin to marine agar plates however masked the original temperature sensitive phenotype at 4°C (Figure 45). The addition of streptomycin and arabinose to marine agar plates reduced the growth of *P. profundum* SS9 strains at 4°C. A reduction in growth was seen with plates containing 1% (w/v) arabinose which had little noticeable growth after 31 days at 4°C (data not shown). At 4°C, growth of mucoid colonies was seen with FL26 pFL190 on 0.1% (w/v) arabinose.

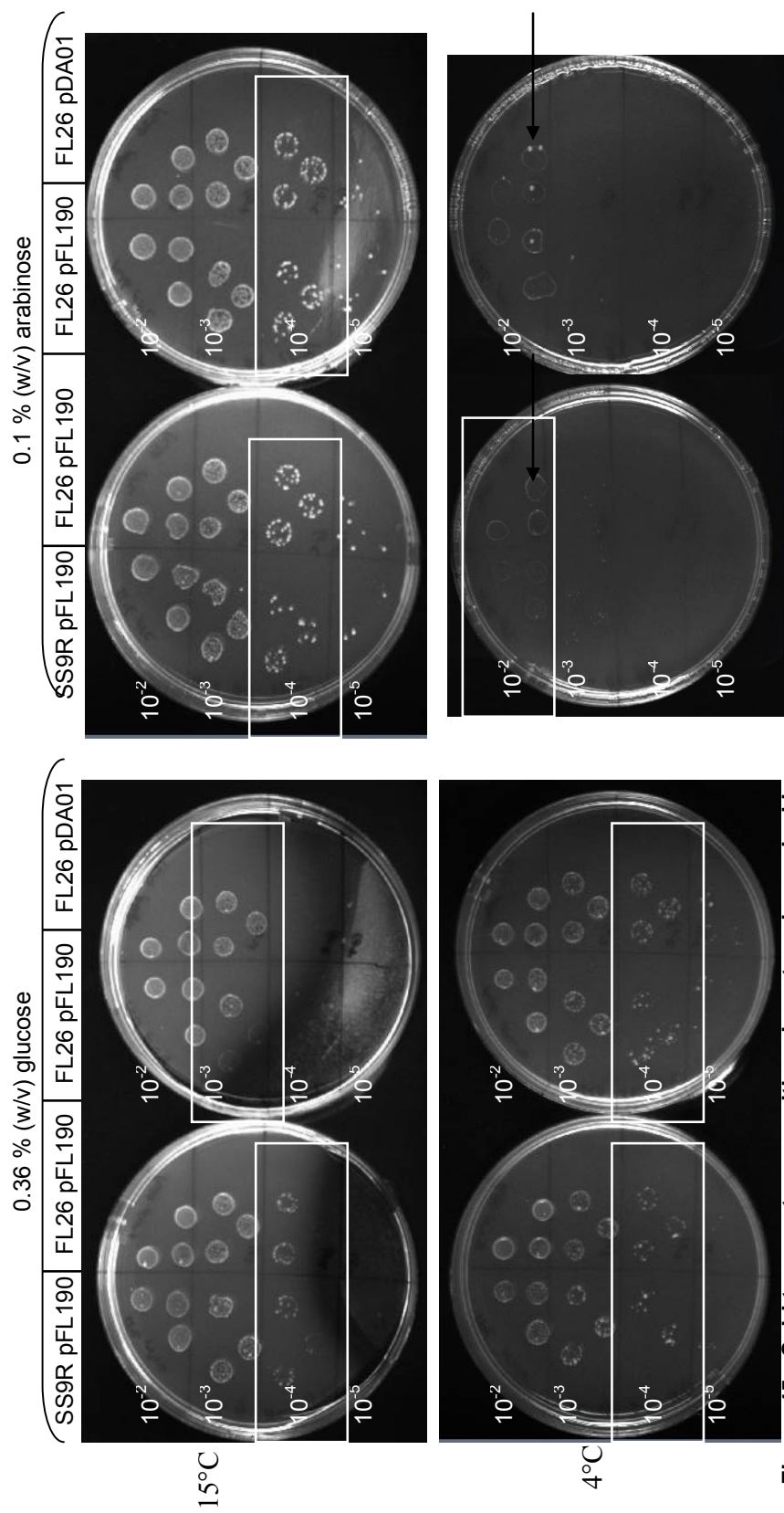


Figure 45: Cold temperature sensitive phenotype is masked by growth on streptomycin

Stationary phase cultures from 15°C (0.1 MPa shaking 200 rpm aerobic) liquid cultures were diluted to an OD₆₀₀ 0.2 in fresh marine broth and serially diluted to 10⁻⁵. These dilutions were spotted in triplicates of 10 µl each onto marine agar plates with 0.36% (w/v) glucose, arabinose where shown and streptomycin. Plates were incubated for 5 days for all plates at 15°C, 14 days for marine agar plates with glucose at 4°C and 31 days for marine agar plates with arabinose at 4°C. Plates incubated with 1% (w/v) arabinose grew poorly and are not shown. Arrow indicates mucoid colonies growth. White boxes highlight the lowest dilution with significant growth in all 3 spots

To test whether pDA01 could complement the LPS alteration, FL26 was grown in shaking aerobic culture at 15°C in a range of arabinose concentrations and the LPS was isolated by SDS-lysis and visualised by SDS-PAGE. While alcian blue silver staining had been used previously to demonstrate the mutant LPS profiles, this method was found to stain too heavily for the complementation. For SDS-PAGE gels investigating the complementation of LPS phenotypes sodium-m-periodate silver staining was used. In the presence of 0.5 and 1.0% (w/v) arabinose FL26 pDA01 had a partial restoration of the smooth LPS band (Figure 46). FL26 pDA01 grown in the presence of 1% arabinose did have a restoration of the smooth LPS band, however this reproducibly had a reduced intensity compared to the parent strain.

The ability of pDA01 to complement the increased motility alteration in FL26 was assessed. Cultures that were not pre-grown in the presence of arabinose were first tested. This showed that FL26 pDA01 still had a significantly greater motility than the parent strain (Figure 47A-C). Cultures were then pre-grown in the presence of arabinose and the motility reassessed. In the presence of 0.1% (w/v) arabinose the motility of FL26 pDA01 was still significantly greater than SS9R pFL190 and FL26 pFL190 (Figure 47D-F). However, the motility at 1% (w/v) arabinose was more problematic because SS9R pFL190 had significantly greater motility than the FL26 pFL190 control (Figure 47F).

It is possible that the O-antigen ligase alone will not complement the mutant phenotypes in FL26, potentially due to downstream effects on *pbpra0217*. To test this hypothesis both *pbpra0218* and *pbpra0217* were amplified together by PCR, digested and cloned into pFL190. However, the sequencing of this construct revealed numerous errors. Unfortunately there was insufficient time to further investigate this.

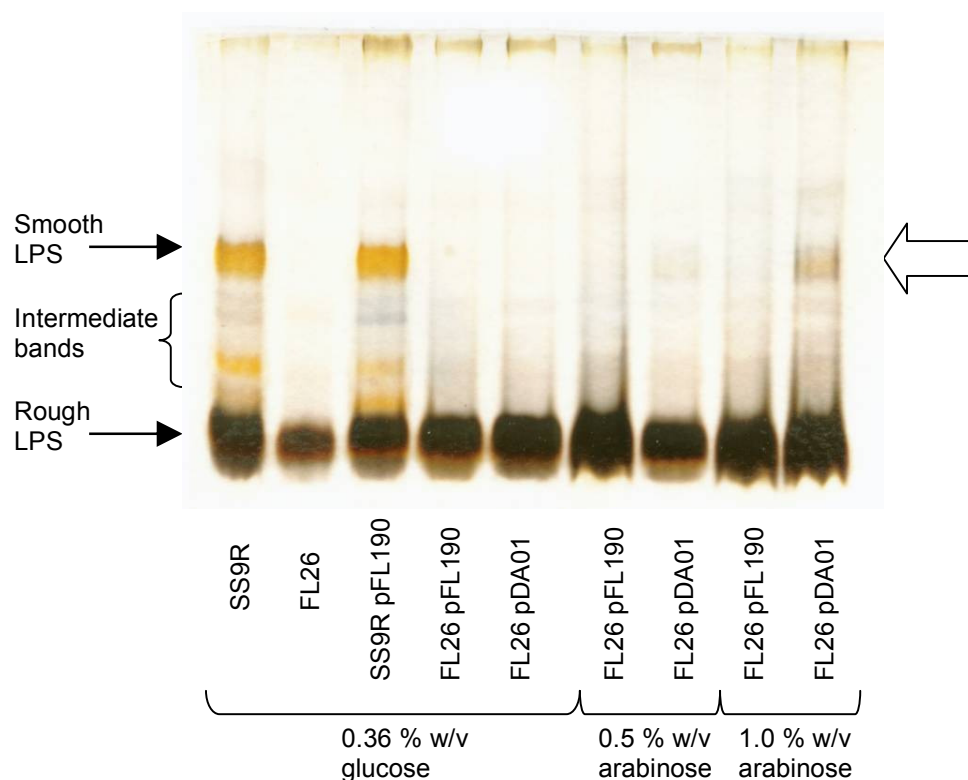


Figure 46: Expression of *pbpra0218* (pDA01) will partially complement the LPS SDS-PAGE profile of FL26

Stationary phase cultures from 15°C (0.1 MPa shaking 200 rpm aerobic) liquid cultures were pelleted by centrifugation. The LPS were isolated by SDS-lysis and then analysed by SDS-PAGE followed by sodium-m-periodate silver staining. *P. profundum* SS9R, SS9R pFL190, FL26, FL26 pFL190 and FL26 pDA01 were grown in the presence of 0.36% w/v glucose and FL26 pDA01 in 0.5% and 1.0% w/v arabinose. White arrow indicates the upper bands on the LPS profile.

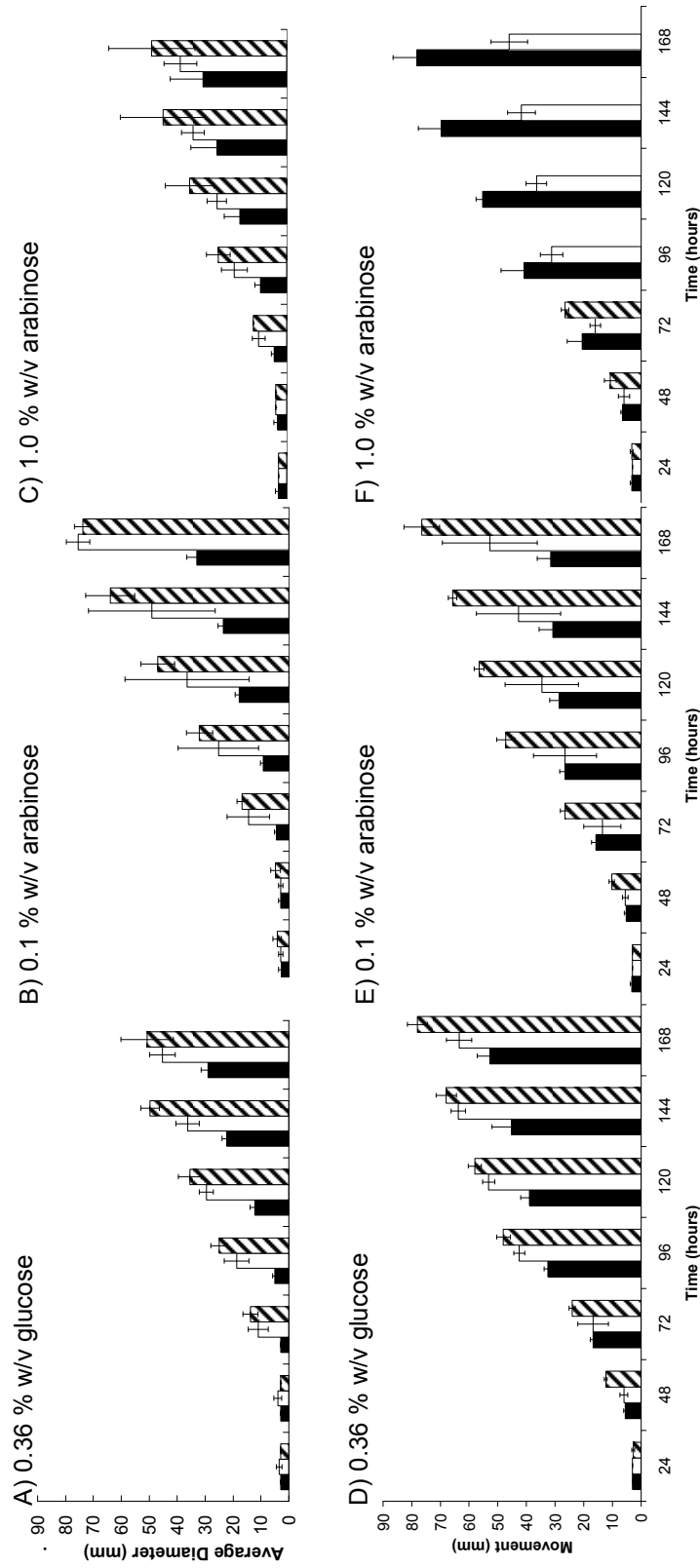


Figure 47: Expression of *pbpra0218* (pDA01) does not complement the motility phenotype of FL26

Anaerobic stationary phase cultures from 15°C (0.1 MPa) (A, B and C) and aerobic cultures from 15°C (0.1 MPa 200 rpm shaking) (D, E and F) were diluted to an OD₆₀₀ 1.0 in fresh marine broth and 3 μ l was spotted onto the centre of a marine motility plate containing 0.3% w/v agar and streptomycin. The plates were then incubated at 15°C and the diameter of measured each day. The average diameter was calculated from 2 motility plates with 2 diameter measurements each. *P. profundum* SS9R pFL190 (filled), FL26 pFL190 (white) and FL26 pDA01 (diagonal). 0.36% w/v glucose (A and D), 0.1% w/v arabinose (B and E) and 1.0% w/v arabinose (C and F). Error bars represent ± 1 SD.

4.7 Complementation of *S. Typhimurium* LT2 SL3749

Due to the large amount of work already performed on *S. Typhimurium* LPS mutants and O-antigen ligase in particular, the ability of to complement the phenotypes in SL3749 were assessed using pFL190. This was in part to ensure that the expression system was capable of complementation and also to test whether SL3749 could be cross-complemented with the *P. profundum* SS9 O-antigen ligase.

4.7.1 Expression of pDA01 (pFL190 *pbpra0218*) will not complement the LPS related phenotypes of SL3749

The plasmid containing the *P. profundum* SS9R O-antigen ligase gene, pDA01, was transformed into SL3749 and assessed for complementation of the LPS profile alteration. SDS-PAGE of the LPS following induction of *pbpra0218* in SL3749 showed that pDA01 did not appear to be able to complement the LPS defect in SL3749 (Figure 48).

The motility assay was repeated to investigate whether the expression of pDA01 can increase the motility of SL3749 on 0.3% (w/v) LB agar plates (Figure 49). pDA01 did not complement this phenotype, however it emerged that expression of pDA01 reduced motility further (Figure 49). After 24 hours incubation the parent strain and SL3749 (both containing empty pFL190) had filled the plates. SL3749 pDA01 had moved 35 mm in the presence of glucose and 24 and 25 mm on 0.1 and 1.0% (w/v) arabinose, respectively. While this result was initially disappointing for not complementing the SL3749 motility phenotype, it did indicate that the *P. profundum* SS9 O-antigen ligase may be performing an action in *S. Typhimurium* LT2, resulting in the reduced motility. It is also possible that the expression of pDA01 in SL3749 is toxic.

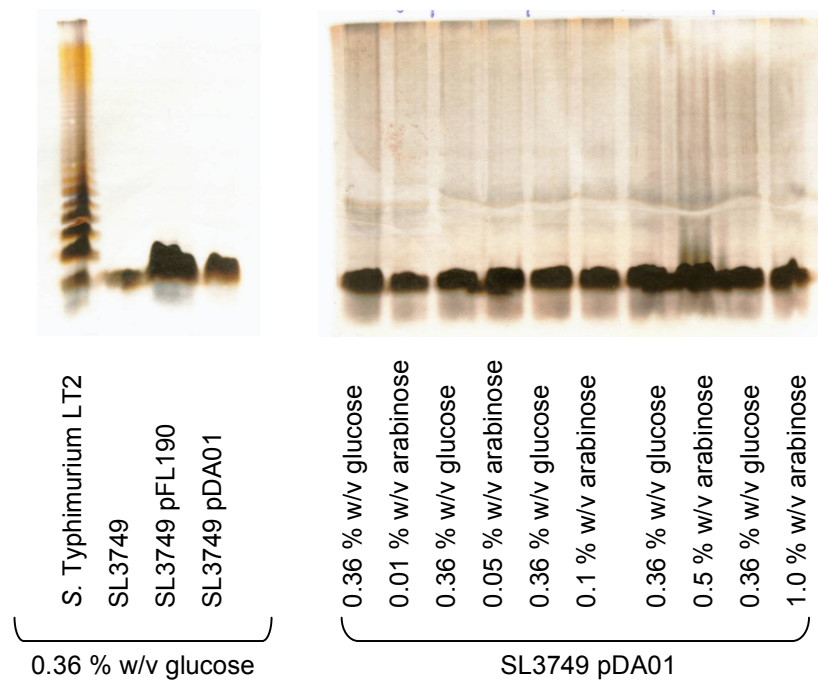


Figure 48: Expression of *pbpra0218* (pDA01) in SL3749 does not complement the LPS profile alteration

Stationary phase cultures from 37°C liquid cultures were pelleted by centrifugation. The LPS were isolated by SDS-lysis and then analysed by SDS-PAGE followed by sodium-m-periodate silver staining. *S. Typhimurium* LT2, SL3749, SL3749 pFL190 and SL3749 pDA01 were grown in the presence of 0.36% w/v glucose and SL3749 pDA01 in 0.01% to 1.0% w/v arabinose.

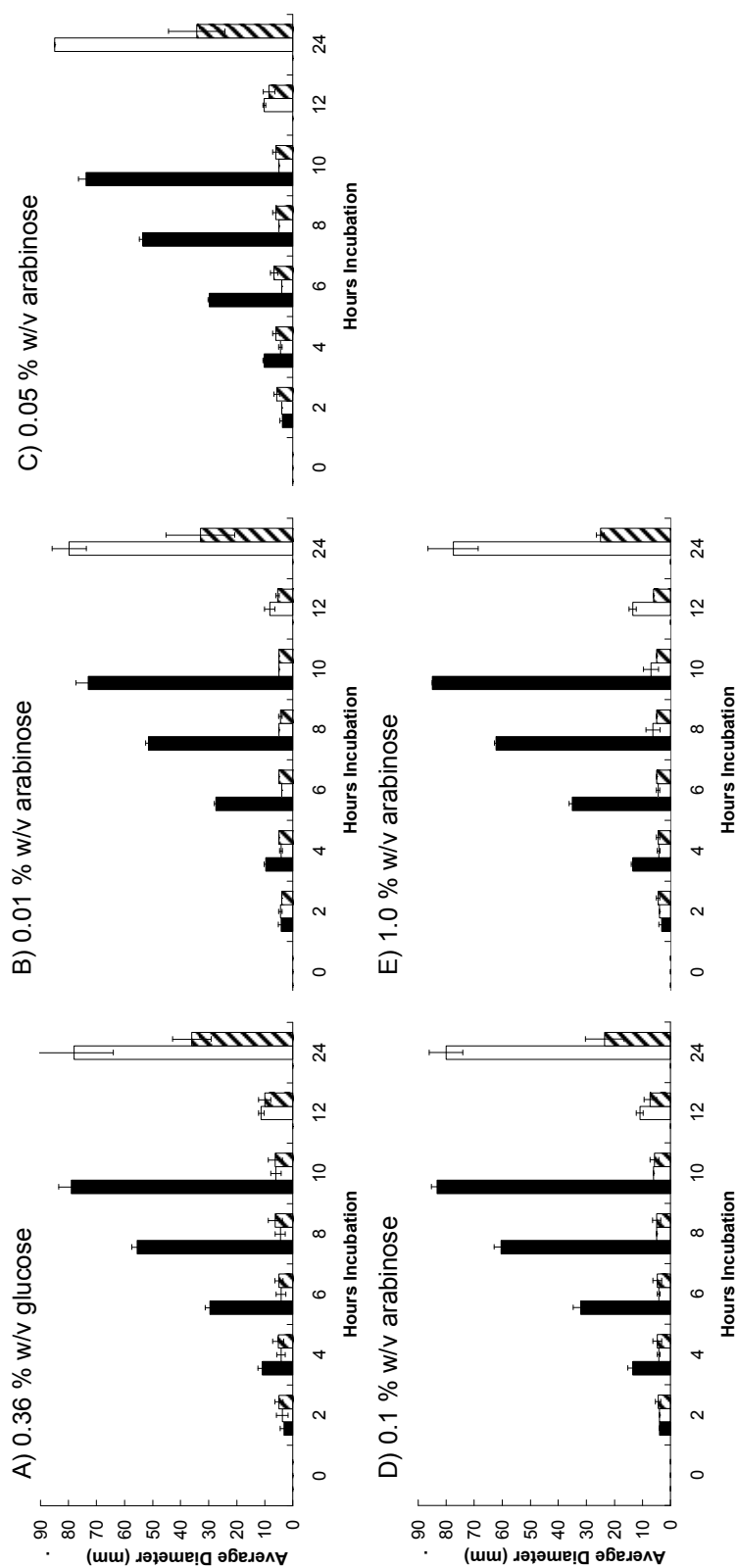


Figure 49: Expression of *pbpra0218* (pDA01) in SL3749 does not complement the reduced motility alteration

Stationary phase cultures were diluted to an OD_{600} 1.0 in fresh LB and 3 μ l was spotted onto the centre of an LB motility plate containing 0.3% w/v agar and streptomycin. The plates were then incubated at 37°C and the diameter of measured each day. The average diameter was calculated from 2 motility plates with 2 diameter measurements each. S. Typhimurium LT2 pFL190 (filled), SL3749 pFL190 (white) and SL3749 pDA01 (diagonal). 0.36% w/v glucose (A), 0.01% w/v arabinose (B), 0.05% w/v arabinose (C), 0.1% w/v arabinose (D) and 1.0% w/v arabinose (E). After 10 hours all of the S. Typhimurium LT2 plates had been completely filled. Error bars represent ± 1 SD.

To ensure that the reduced motility was not a result of a reduced growth rate, the growth of SL3749 pDA01 was compared to SL3749 pFL190 in LB alone (Figure 50). The growth was identical, therefore showing that the difference in motility was not due to a reduction in growth.

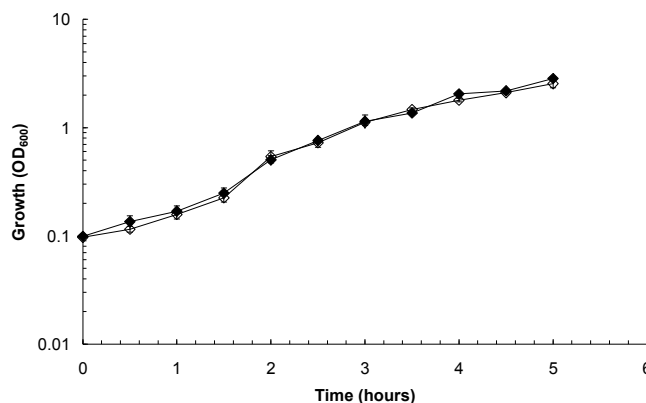


Figure 50: Growth of SL3749 is not affected by expression of *pbpra0218* (pDA01)

Stationary phase cultures were diluted to an OD₆₀₀ 0.1 in fresh LB streptomycin and incubated at 37°C 200 rpm. SL3749 pFL190 (♦) and SL3749 pDA01(◇).

4.7.2 Expression of pDA03 (pFL190 *S. Typhimurium* LT2 O-antigen ligase) will complement the LPS related phenotypes of SL3749

SL3749 has been previously complemented using the *Salmonella* SARC 1 O-antigen ligase gene contained on pWQ310 (Kaniuk *et al.*, 2004). This was digested from pWQ310 using *EcoRI* and *XbaI*, cloned in pFL190 and transformed into *E. coli* DH5α. The transformants were screened by restriction digest and the clone containing the correct construct, pDA03, was then transformed into SL3749 (see Appendix C). SL3749 pDA03 was grown in the presence of a range of arabinose concentrations and the LPS profile was shown to be restored to the parent profile under all arabinose concentrations tested (Figure 51).

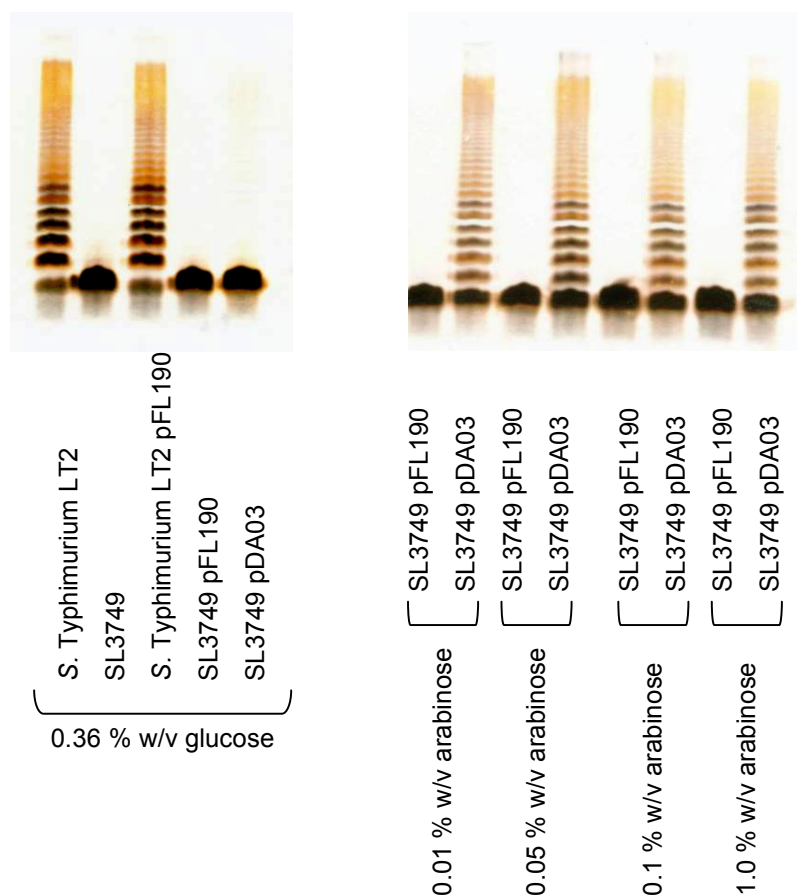


Figure 51: SDS-PAGE of LPS profile of SL3749 expressing *S. Typhimurium* LT2 O-antigen ligase (pDA03)

Stationary phase cultures from 37°C liquid cultures were pelleted by centrifugation. The LPS were isolated by SDS-lysis and then analysed by SDS-PAGE followed by sodium-m-periodate silver staining. *S. Typhimurium* LT2, *S. Typhimurium* LT2 pFL190, SL3749, SL3749 pFL190 and SL3749 pDA03 were grown in the presence of 0.36% w/v glucose and SL3749 pDA03 in 0.01% to 1.0% w/v arabinose.

SL3749 pDA03 was also tested for complementation of the motility phenotype. On all plates, the parent strain still had a significantly greater motility than SL3749 pFL190 and SL3749 pDA03 (Figure 52). In the presence of arabinose SL3749 pDA03

displayed an improved motility, close to that of the parent (Figure 52B, C and D). In the absence of arabinose, SL3749 pDA03 again displayed partial complementation with an increased motility compared to the empty plasmid control (Figure 52A). However, the complementation was incomplete because there was still a significant difference between the parent and complemented mutant strain. It is likely that pre-growth in arabinose before the motility assay could further reduce the difference between the parent and complemented mutant.

It was therefore demonstrated that the pFL190 expression system was fully functional, complementing the SL3749 LPS profile alteration and at least partially restoring the motility.

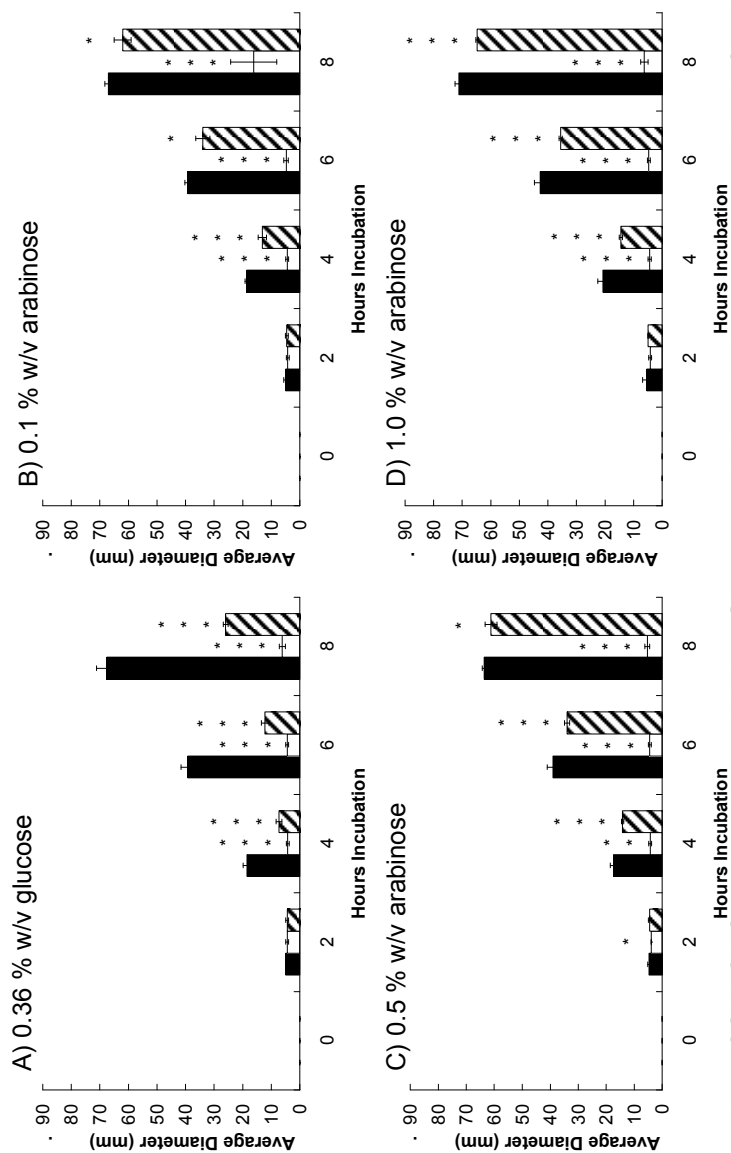


Figure 52: Expression of SARC1 O-antigen ligase (pDA03) SL3749 partially complements the motility defect in SL3749

Stationary phase cultures were diluted to an OD_{600} 1.0 in fresh marine broth and 3 μ l was spotted onto the centre of an LB motility plate containing 0.3% w/v agar and streptomycin. The plates were then incubated at 37°C and the diameter of measured each day. The average diameter was calculated from 3 motility plates with 2 diameter measurements each. S. Typhimurium LT2 pFL190 (filled), SL3749 pFL190 (white) and SL3749 pDA03 (diagonal). 0.36% w/v glucose (A), 0.1% w/v arabinose (B), 0.5% w/v arabinose (C), 1.0% w/v arabinose (D). After 10 hours all of the S. Typhimurium LT2 plates had been completely filled and were no longer plotted. The mean motility was compared with S. Typhimurium LT2 using the unpaired student's t-test, 2 tailed: $P > 0.05$ (no symbol), $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***). Error bars represent ± 1 SD.

4.8 Discussion

4.8.1 The *P. profundum* SS9 gene, *pbpra0218*, encodes an O-antigen ligase

The *P. profundum* SS9R mutant strain FL26 contains a mini-Tn5 transposon in *pbpra0218*, a gene annotated as a putative O-antigen ligase. This chapter has demonstrated by SDS-PAGE and DOC-PAGE that the LPS profile of FL26 shares similarities with O-antigen ligase mutants from other bacteria such as *V. cholera*, *E. coli*, *P. aeruginosa* and *S. enterica* serovar Typhimurium. FL26 has lost a major upper band and most intermediate bands, corresponding to smooth LPS and varying states of O-antigen polymerisation. Given the LPS profile alteration and the similarity to *V. fischeri*, O-antigen ligase, *pbpra0218* does appear to encode for an O-antigen ligase.

Since FL26 is an O-antigen ligase mutant, GC-MS analysis was used to investigate differences in carbohydrate composition. FL26 had a significant reduction in mannose and galactose compared to the parent, and lacked trace amounts of xylose and arabinose that were present in the parent strain. Mannose, galactose and arabinose have all been previously demonstrated to make up the O-antigen of various *V. cholerae* serotypes (Chatterjee and Chaudhuri, 2003). This suggested that the *P. profundum* SS9 O-antigen consists of mannose and galactose, possibly with traces of xylose and arabinose.

The fatty acid composition was analysed to investigate whether other alterations had occurred in the LPS. The fatty acid composition indicated that FL26 had a reduction in the amount of C14:OH and C16:0. It was also shown that there were some minor increases in C12:0 and C12:OH. These alterations could be indicative of further changes to the FL26 LPS composition as an adaptive process to cope with the loss of O-antigen. It has been previously shown that longer chain fatty acids are linked to increased membrane fluidity (Sinensky, 1971). The decrease in the levels of C16:0 and C14:OH, and the reciprocal increase in C12:0 and C12:OH might indicate a less viscous membrane and be a possible explanation for the cold temperature sensitivity. The

psychrophilic bacterium *P. syringae* accumulated hydroxylated fatty acids cold temperatures (Kumar *et al.*, 2002) and piezophiles have been demonstrated to have higher levels unsaturated fatty acids (UFA's) (Allen *et al.*, 1999; Fang *et al.*, 2003).

4.8.2 FL26 displays a cold temperature sensitive phenotype on marine agar

As demonstrated, FL26 displayed a cold temperature sensitivity on solid media that was not reproduced in liquid media, consistently growing to a serial dilution 10 fold less than the parent strain at 4°C (0.1 MPa). This was shown not to be a general growth defect as continued incubation did not increase the growth on marine agar and because liquid growth curves revealed that FL26 had a very similar growth to the parent strain. 2D gel analysis of proteins expressed under cold shock by *E. coli* K12 had shown that the protein patterns of immobilised cells and suspended cells differ (Frédérique Perrot, 2001). This could mean that cells experience cold temperature differently depending on the nature of the media they are in, and that LPS alterations (which are well documented to increase sensitivity to certain cell envelope) may enhance this effect. This work suggests that O-antigen ligase may be necessary to perform the LPS modification seen in *P. profundum* SS9R when grown on agar or from temperature changes, and consequently, that these are changes in the O-antigen. Microarray data shows that O-antigen ligase is down-regulated at 4°C (Campanaro *et al.*, 2005; Vezzi *et al.*, 2005), which makes the explanation for O-antigen ligase activity and its requirements more complicated. Although this data has not yet been followed up by RT-PCR.

When discussing the cold temperature sensitive phenotype it is also important to consider the methodology by which this was measured. Cultures were pre-grown anaerobically at 15°C (0.1 MPa), then diluted to a specified OD₆₀₀ before being incubated at 4°C (0.1 MPa) on marine agar plates. It would have been an interesting addition to test whether the same reduction in growth occurred if the pre-cultures were grown at 4°C (0.1 MPa).

Complementation of the cold temperature sensitivity was problematic. Growing cultures to the same phase of growth was troublesome because in practice *P. profundum* SS9 strains containing plasmids were slower to grow than those without (data not shown) making comparisons difficult. Care had to be taken in the pre-growth of cultures for complementation assays and eventually reproducible data was recorded. Through numerous attempts it was found that the cold temperature sensitive phenotype of FL26 was masked following transformation with pFL190. This was unfortunate as it would make assessing any complementation impossible. FL26 pFL190 and SS9R pFL190 on marine agar containing glucose and streptomycin, incubated at 15°C (0.1 MPa) showed similar growth with both strains having significant growth at 10⁻⁴ dilution. This indicated that the cultures were at least comparable at 15°C. Identical plates incubated at 4°C (0.1 MPa) showed no temperature sensitive phenotype on marine agar glucose streptomycin plates as both cultures again had significant growth at 10⁻⁴ dilution. FL26 pFL190 and SS9R pFL190 were incubated at 15°C on marine agar plates containing glucose, streptomycin and 0.1% (w/v) arabinose and growth was seen at 10⁻⁴ dilution. This was similar to those plates without arabinose with the exception that plates containing arabinose took 9 days longer for significant growth. At 4°C, a large reduction in growth was seen when arabinose was added to the marine agar plates. Minimal growth was found on 0.1% (w/v) arabinose plates after 31 days incubation and the colonies appeared mucoid. Marine agar plates which contained 1% (w/v) arabinose showed no significant growth after 40 days incubation at 4°C. It seemed that the presence of arabinose under the cold temperature conditions was detrimental to the growth of *P. profundum* SS9R.

Additionally, attempts were made to assess the complementation by growth on marine agar plates lacking streptomycin. These plates had a greater level of variability than those which containing streptomycin. Also, the extended periods of incubation required to assess growth in the presence of arabinose at 4°C resulted in large amounts of fungal contamination.

It is likely that some of the phenotypes seen may be related to the gene downstream from *pbpra0218*, *pbpra0217*. While it is believed that mini-Tn5

transposons should not have downstream effects (Larsen *et al.*, 2002), *pbpra0218* and *pbpra0217* overlap by 6bp, and therefore the possibility of downstream effects should not be ignored.

4.8.3 FL26 displays an LPS profile alteration by SDS-PAGE that can be partially complemented

The LPS profile alteration in FL26 is similar to that seen in other O-antigen ligase mutants from various backgrounds. FL26 has a reduction in the smooth LPS and loss of intermediate bands. The LPS profile was partially restored by expression of *pbpra0218* on pFL190 (pDA01), in the presence of 0.5 and 1.0% (w/v) arabinose the smooth LPS band is partially restored to parent levels. It could be likely that the partial complementation is due to effects on the downstream gene *pbpra0217* which has a 6 bp overlap with *pbpra0218*. Attempts to clone both genes were unsuccessful. While a construct was created by PCR of both genes and cloned into pFL190 (pDA04), the sequencing of this construct revealed a large amount of errors. This may be explained if the gene had toxic effects in the *E. coli* DH5 α cloning strain. The effect of expressing both genes in FL26 was therefore not investigated at this time.

The complementation system was shown to be effective when the *S. Typhimurium* LT2 O-antigen ligase gene was successfully cloned into pFL190 (pDA03) and expressed in SL3749. This fully restored the parent LPS profile under all arabinose concentrations tested. The *P. profundum* SS9 O-antigen ligase was shown not to cross complement when pDA01 failed to restore a parent LPS profile in SL3749.

While O-antigen ligases tend to share low sequence similarity the secondary structure can usually reveal a relationship. The reason for the low sequence homology has been postulated to be due to the high substrate specificity that O-antigen ligases demonstrate (Kaniuk *et al.*, 2004). This is apparent in attempts to cross-complement, i.e. cloning a similar gene from one bacterium into another, and vice-versa. Studies have shown difficulties in cross-complementing O-antigen ligases from different species, such as between and *P. aeruginosa* and *E. coli* (Abeyrathne and Lam, 2007). Complementing within a species is usually successful (Abeyrathne *et al.*, 2005; Kaniuk

et al., 2004). This was borne out in this study, where it has been shown that expression of *pbpra0218* in SL3749 did not complement the LPS alteration phenotype.

4.8.4 FL26 displays an increased motility compared to the parent strain

FL26 was demonstrated to have a significantly increased motility compared to the parent strain. Expression of pDA01 did not complement the increased motility phenotype of FL26. Initially *P. profundum* SS9 cultures were grown anaerobically and used to inoculate motility plates containing a range of arabinose concentrations. With the finding that FL26 has a partial restoration on the LPS profile when grown in shaking culture, the cultures for the motility complementation were pre-grown in the presence of arabinose in shaking culture. However, this still did not complement the motility alteration.

The *S. Typhimurium* O-antigen ligase mutant, SL3749, also showed an alteration in motility on 0.3% (w/v) LB agar plates, albeit the opposite to FL26. SL3749 showed a reduction in motility compared to the parent strain. The LPS of SL3749 was successfully complemented with the native O-antigen ligase (pDA03) which lead to restoration of the LPS profile and a partial restoration of the motility phenotype.

S. Typhimurium LPS mutants have previously been shown to have a wild type swimming motility and reduced swarming motility (Toguchi *et al.*, 2000). While this may contradict with previously published results it is necessary to point out that the exact composition of the LB swim plates differed to those used in this study. Previous work has shown that flagellin levels of SL3749 were not significantly altered compared to the parent strain (Toguchi *et al.*, 2000). Again, while this data may appear to conflict with previously published results it is important to highlight that the quantification of flagella in the paper was measured from cells grown on swarming plates. Our data show images of bacteria from liquid cultures because bacteria could not be successfully isolated back from the swimming plates (i.e. bacteria move through the agar in swimming plates, whilst they move on top of the agar in swarm plates).

4.8.5 Cold temperature sensitive LPS biosynthesis mutants of *P. profundum* SS9 also have an increased motility

The motility defect in O-antigen ligase mutants has been well documented and indeed in FL26 there is a motility alteration compared to the parent strain, *P. profundum* SS9R. However, this was seen as a significantly increased motility. This increased motility was also demonstrated in several other *P. profundum* SS9 LPS biosynthesis mutants. To explore the reason for this, the flagella structure was investigated because previous work had shown that the reduced motility in *P. aeruginosa* O-antigen ligase mutants was due to a complete lack of flagella and pilli (Abeyrathne *et al.*, 2005). Rather than a lack of flagella, it was postulated that the difference in the motility of *P. profundum* SS9 LPS mutants may be due to the multiple flagella systems present in *P. profundum* SS9 which are differentially expressed at 0.1 MPa and 28 MPa (Vezzi *et al.*, 2005). A recent paper has shown that the 2 flagella systems work under differently viscous environments, peritrichious flagella under high viscosity and a polar single flagellum under low viscosity (Eloe *et al.*, 2008). It seemed reasonable to assume that alterations in the membrane may affect pressure sensing and result in the bacteria expressing the wrong flagella system, hence making FL26 move significantly faster. The first method to visualise the flagella was a simplified Leifson's flagella stain, which involves loading dye onto the flagella so that it might be seen by light microscopy. This method resulted in very few visible flagella and was too variable to be relied upon. The inability to be detected by Leifson's stain may indicate something about the composition of the *P. profundum* SS9 flagella, since the stain and procedure work adequately for various other strains including *V. cholera* (Clark, 1976) and for the *Salmonella* strains investigated in this chapter. When TEM was utilised a large number of flagella were found, however the flagella were commonly damaged or incomplete, most likely due to the processing of samples. There was also variability in the number of flagella visualised and because the flagella were often damaged it was difficult to identify which bacterium they originated from.

In an on-going collaboration with the Scripps Institute of Oceanography, University of California, USA, another method to visualise the flagella was used. A NanoOrange fluorescent dye which binds proteins was employed and vividly displayed that *P. profundum* SS9R and FL26 taken from agar plates produced 1 uniform polar flagellum. Again, it may be that the precise conditions for swimming motility must be used to visualise the flagella, or that due to the fragile nature of peritrichious unsheathed flagella, they are simply impossible to visualise. Purification of the flagella may be the only method to rule out alterations. While 2 potential flagella systems exist in the *P. profundum* SS9 genome, the expression of the peritrichious flagella has not yet been proved by purification of visualisation. Peritrichious flagella are responsible for swimming and swarming phenotypes (Harshey, 2003), however unsheathed flagella are notoriously fragile and difficult to isolate (Allen and Baumann, 1971; McCarter, 2001).

V. cholera is closely related to *P. profundum* SS9, and by using immuno-gold labelling TEM demonstrated that *Vibrio cholera* has a sheathed flagella (Fuerst and Perry, 1988) and indeed a variety of *Vibrionaceae* also have sheathed flagella (McCarter, 2001). The flagellum shared LPS antigens with the rest of the cell and it has been shown that the sheath is an extension of the outer membrane. This may form a tenuous hypothesis as to why the LPS alteration gave a motility phenotype. If the loss of O-antigen also resulted in a change in the sheath of the flagellum possibly making the flagellum faster or more efficient. Further work must be performed on the *P. profundum* SS9 flagella, including whether it is sheathed, the mechanism of motility and actual swimming speeds of the mutant strains.

4.8.6 Summary and conclusions

The phenotypes arising from the transposon insertion in the O-antigen ligase of *P. profundum* SS9, *pbpra0218*, could be directly linked to the LPS alteration. This could be through a reduction in the stability of the membrane by a reduction in viscosity or through an increase in the permeability of the membrane. This is most likely the reason for the cold temperature sensitivity. Alterations in motility can also be due to the

membrane composition having an increased “wetability”, i.e. making the movement through the medium more efficient. As suggested above, if *P. profundum* SS9 possessed a sheathed flagella, alterations in the LPS may directly affect the function of the flagella.

The other phenotypes may result from a more complex mechanism. LPS alterations are also documented to have effects on the protein composition of the outer membrane (Ames *et al.*, 1974). If these proteins were involved in signalling it is feasible that this could lead to the motility alterations in FL26 through an alteration in chemotaxis. If the complementation of the LPS is only partial, the outer membrane protein composition could still be affected, explaining why the motility alteration was not complemented.

5. Results – Characterisation of the growth and LPS of a putative *P. profundum* SS9 tyrosine protein kinase mutant, FL9

5.1 Introduction – tyrosine protein kinase, Wzc

The mutant *P. profundum* SS9 strain, FL9, was isolated from a screen for cold temperature sensitive mutants following a random transposon mutagenesis (Lauro *et al.*, 2008). FL9 contains a mini-Tn5 transposon insertion in *pbpra2686*, a putative tyrosine protein kinase (*wzc*) which, in *E. coli*, is required for the expression of a group 1 or colanic acid capsule (Whitfield, 2006) (Figure 53 and Table 18). In *E. coli*, the tyrosine protein kinase Wzc forms a part of a complex with Wza, Wzb, Wzx and Wzy. Wzc has been demonstrated to be required for the polymerisation and transport of CPS to the outer membrane in *E. coli* (Reid and Whitfield, 2005). Wzx flips the capsule subunits (und-pp-linked) across the inner membrane, where the polymerase autophosphorylation of Wzc and the phosphatase activity of Wzb, along with Wzy, interact to ensure polymerisation of CPS (Drummelsmith and Whitfield, 1999; Nesper *et al.*, 2003). Wzc and Wza form a channel across the periplasm which permits the transport of CPS to the outer membrane (Drummelsmith and Whitfield, 2000; Reid and Whitfield, 2005).

E. coli with mutations in genes encoding Wzc have been shown to have an altered capsule by one of two methods: a reduced staining with cationised ferritin compared to the parent strain by TEM (Reid and Whitfield, 2005), or a reduced detection of CPS by western blot (Paiment *et al.*, 2002). In the absence of Wzc, *E. coli* strains that form a group 1 capsule continue to synthesise the capsular polysaccharides, although these are assembled on the cell surface as KLPS (Drummelsmith and Whitfield, 1999; Wugeditsch *et al.*, 2001) (Figure 54). The effects of a mutation in Wzc have been characterised in *E. coli* as a loss of capsule and an increase in the expression of K_{LPS}.

This chapter describes the characterisation of a putative tyrosine protein kinase mutant, FL9, and the phenotypes that arose from the transposon insertion. Growth under cold temperatures and high pressures, and the structure and expression of LPS and CPS were investigated.

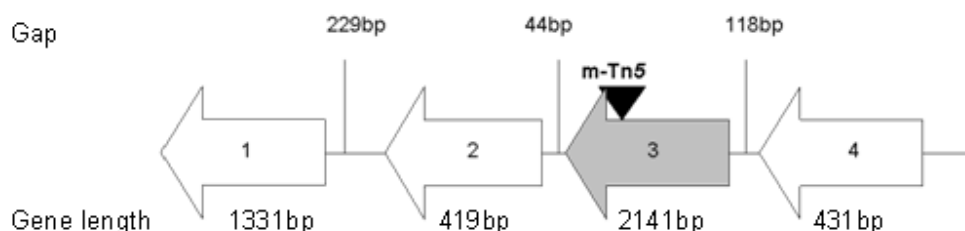


Figure 53: Graphical representation of gene cluster. FL9 contains a mini-Tn5 transposon insertion in *pbpra2686* which encodes a putative tyrosine protein kinase, Wzc

The proteins encoded by the above genes are described in Table 18, along with relative identity and similarity to previously described proteins.

Table 18: Genome annotation and protein homology in *P. profundum* SS9 Chr1: 3113782-3118495

	Genome Annotation	Proposed Function	Closest Biochemically Proven Homolog
1	<i>pbpra2684</i>	Wzx	34% identity (57% similarity) <i>Mannheimia succiniciproducens</i> MBEL55E
2	<i>pbpra2685</i>	Lob1 Glycosyltransferase	67% identity (80% similarity) <i>Shewanella sediminis</i> HAW-EB3
3	<i>pbpra2686</i>	Wzc	58% identity (76% similarity) <i>Vibrio vulnificus</i> YJ016
4	<i>pbpra2687</i>	cytoplasmic phosphatase	70% identity (86% similarity) <i>Vibrio vulnificus</i> YJ016

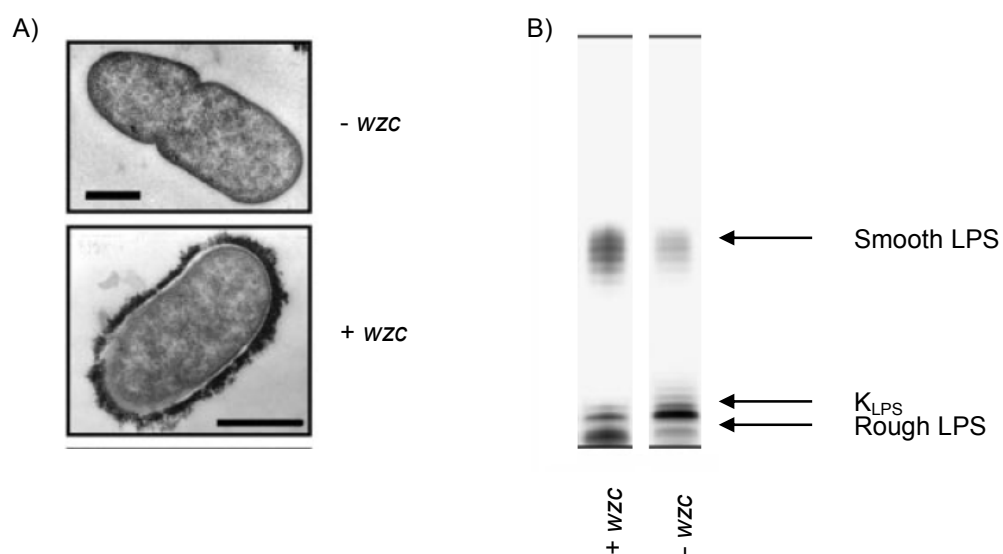


Figure 54: Mutations in *wzc* reduce CPS expression and cause a change in the LPS profile by Tricine-PAGE

TEM images show *E. coli* CWG285 (*wzc* mutant) and CWG285 pWQ130 (complemented) stained with cationised ferritin (A) (Reid and Whitfield, 2005). *E. coli* CWG258 (parent strain) and CWG285 (*wzc* mutant) LPS visualised by Tricine-PAGE and silver staining (B) (Wugeditsch *et al.*, 2001). Bar equals 0.5 μm .

5.2 Investigating the cold temperature sensitivity of FL9 relative to the parent strain, SS9R

FL9 was isolated from the same screen for cold temperature sensitive mutants as FL26 (discussed in Chapter 4) which was based on reduced growth on marine agar plates at 4°C compared to the parent strain (Lauro *et al.*, 2008). This section describes the effects of this mutation on the physiology of FL9, specifically on the cold temperature sensitive phenotype on marine agar, in liquid broth and at high pressures.

5.2.1 FL9 displays a cold temperature sensitive phenotype on marine agar

In order to more quantitatively measure the cold temperature sensitive phenotype of FL9, the growth on marine agar was investigated. *P. profundum* cultures were pre-grown to stationary phase at 15°C (0.1 MPa) and diluted to OD₆₀₀ 0.2. Serial dilutions were prepared and spotted in triplicate onto marine agar plates. The plates were incubated at 15 and 4°C (0.1 MPa). At 15°C (0.1 MPa), FL9 had similar growth to the parent strain, reaching 10⁻⁴ dilution (Figure 55). At 4°C (0.1 MPa) FL9 had a 10 fold reduction in growth, reaching 10⁻³. This experiment confirmed that FL9 displays cold temperature sensitive growth on marine agar plates at 4°C (0.1 MPa) compared to the parent strain. Since the growth of FL9 was identical to that of *P. profundum* SS9R at 15°C (0.1 MPa), this indicated that FL9 has a cold temperature sensitive phenotype rather than having a general growth defect.

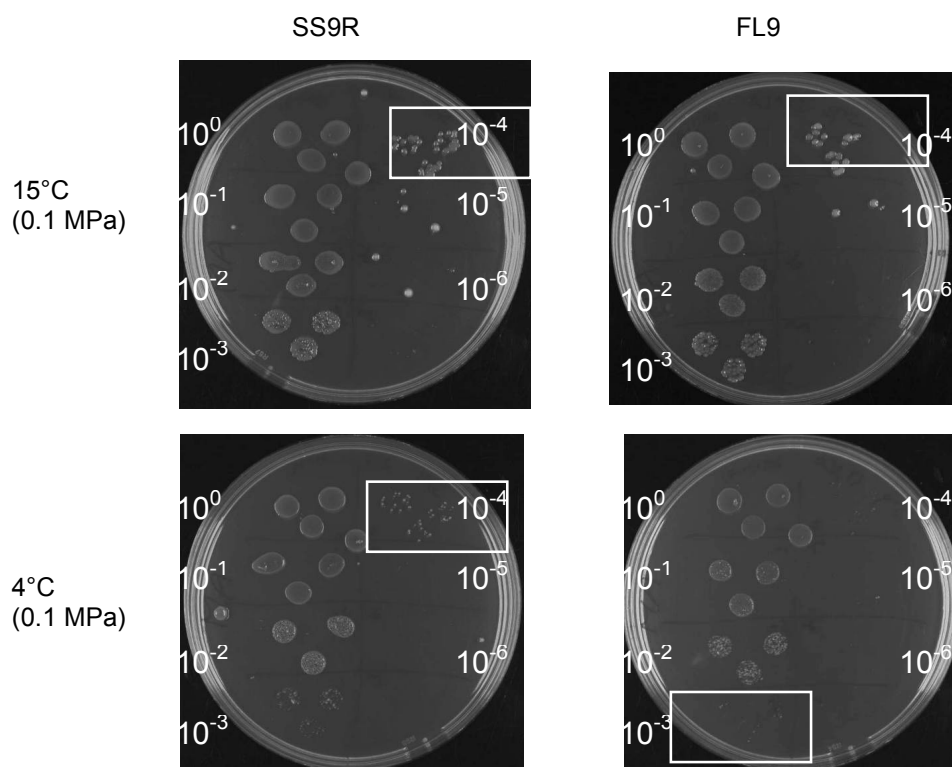


Figure 55: FL9 displays a temperature sensitive growth defect on marine agar at 4°C

Anaerobic stationary phase cultures of *P. profundum* SS9R and FL9 from 15°C (0.1 MPa) were diluted to an OD₆₀₀ 0.2 in fresh marine broth and serially diluted to 10⁻⁶. These dilutions were spotted in triplicates of 10 µl each onto marine agar plates containing 20 mM glucose. The plates were incubated at 15°C and 4°C (0.1 MPa) for 10 days. White boxes highlight the lowest dilution with significant growth.

5.2.2 FL9 does not display a cold temperature sensitive phenotype in marine broth

Cold temperature sensitive mutants were selected for based on reduced growth on marine agar at 4°C (MPa) compared to the parent strain (Lauro *et al.*, 2008). To more fully describe the growth of FL9 in marine broth, stationary phase cultures that were pre-grown at 15°C (0.1 MPa) were diluted to OD₆₀₀ 0.1 and incubated at 15 and 4°C (0.1 MPa). At 15°C (0.1 MPa), FL9 had very similar growth to the parent strain up

until entry into stationary phase at 22 hours (Figure 56A). At 4°C (0.1 MPa) the growth rate was reduced compared to 15°C (0.1 MPa) and both cultures showed similar growth (Figure 56B). This indicated that FL9 did not display a significant alteration in growth in marine broth compared to the parent strain. Therefore the cold temperature sensitive phenotype was only detectable on solid marine agar (Figure 55).

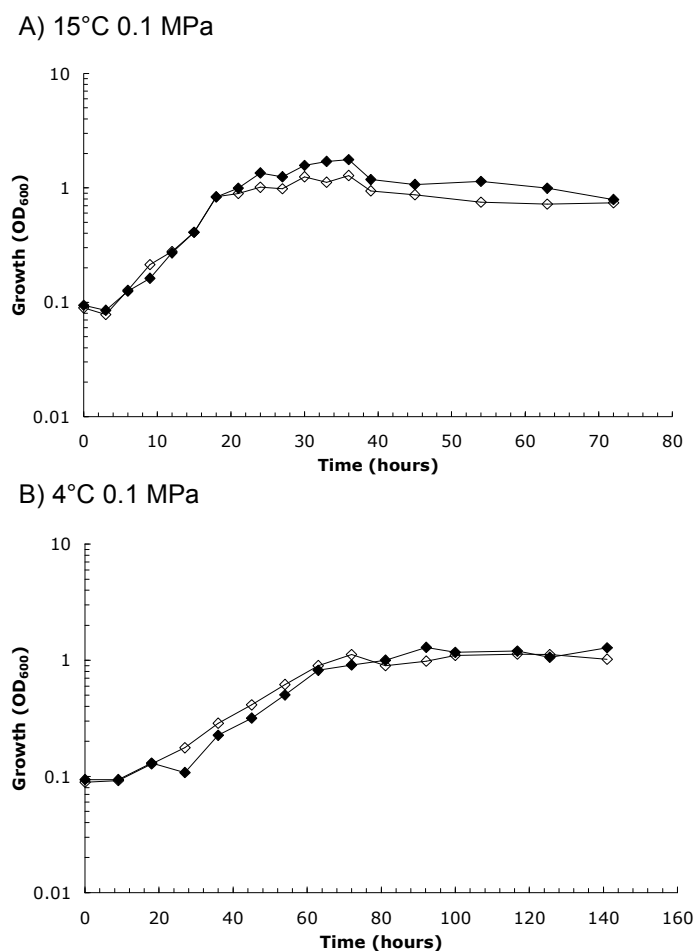


Figure 56: *P. profundum* SS9R and FL9 grow similarly in marine broth at 15°C and 4°C (0.1 MPa)

Anaerobic stationary phase cultures from 15°C (0.1 MPa) were diluted to a starting OD₆₀₀ 0.1 in fresh marine broth with 20 mM glucose and heat sealed in sterile plastic pouches (anaerobic). These were then incubated at the defined conditions. *P. profundum* SS9R (♦) and FL9 (◊). The experiment was performed in duplicate. The above graphs are representative growth curves.

5.2.3 FL9 does not show a high pressure growth alteration using marine agar overlay plates

Due to the fact that the FL9 displayed a temperature sensitive phenotype on marine agar and not in marine broth, the growth on marine agar was also assessed at elevated pressures to investigate whether FL9 also had a pressure sensitive phenotype. Using the method developed in Chapter 3.2.3, stationary phase cultures were diluted to OD₆₀₀ 0.8, serially diluted and spotted in triplicate onto LTGMA plates. These plates were incubated for 2 hours at 15°C (0.1 MPa) and then overlaid with molten LTGMA.

The non-overlaid LTGMA plates were used to demonstrate that FL9 and the parent strain had similar numbers of bacteria before being overlaid with molten LTGMA, with similar growth at 10⁻⁴ dilution (Figure 57). Plates that were overlaid and incubated at 0.1 MPa (15°C) had significant growth at 10⁻³ dilution. After incubation at 28 MPa (15°C), FL9 and the parent strain had significant growth at 10⁻⁴ dilution. Both the mutant and parent strain showed increased growth at 28 MPa compared to 0.1 MPa (15°C). Using this method, FL9 did not show a pressure sensitive phenotype. However, higher pressures should be investigated in future and indeed cold temperatures and high pressures to investigate the relationship between the two.

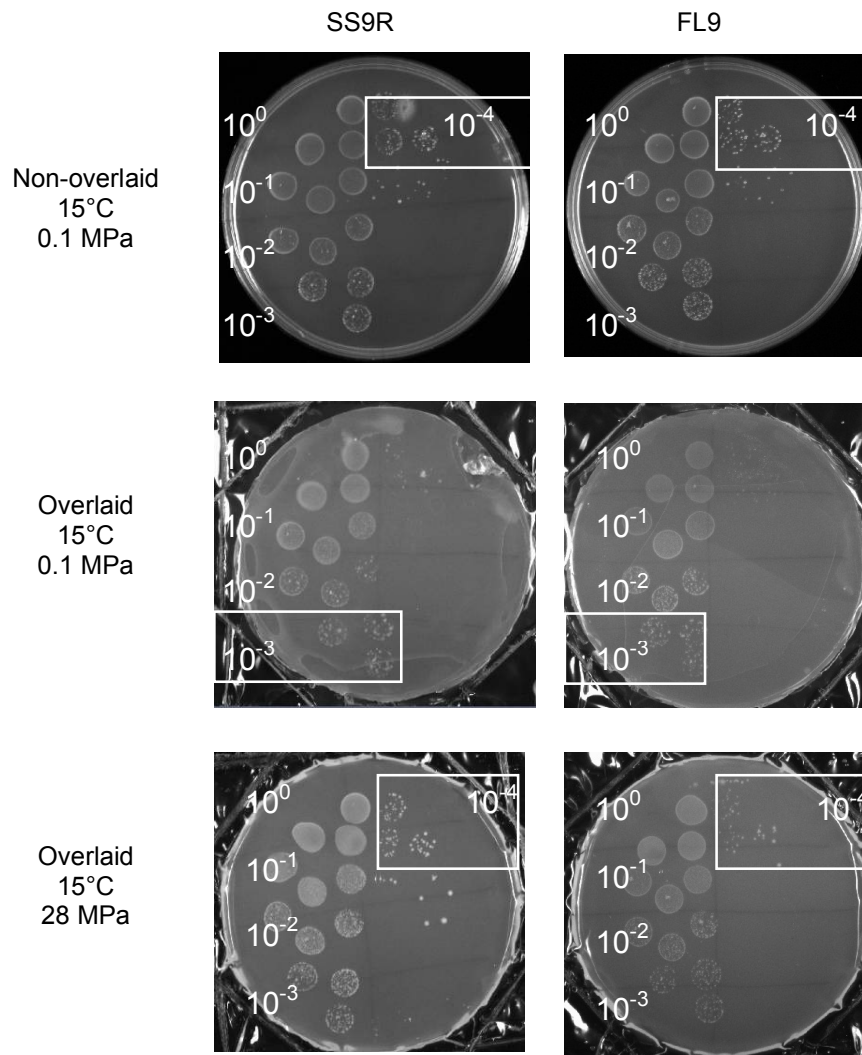


Figure 57: FL9 does not display a high pressure sensitive growth defect on marine agar

Anaerobic stationary phase cultures of *P. profundum* SS9R and FL9 from 15°C (0.1 MPa) were diluted to an OD₆₀₀ 0.8 in fresh marine broth and serially diluted to 10⁻⁶. These dilutions were spotted in triplicates of 10 µl each onto low temperature gelling marine agar (LTGMA) plates containing 20 mM glucose. The plates were incubated at 0.1 MPa 15°C for 2 hours before being overlaid with molten LTGMA and sealing in sterile plastic bags. The plates were then incubated at 0.1 and 28 MPa (15°C). After 6 days the growth was assessed. White boxes highlight the lowest dilution with significant growth in all 3 spots.

5.3 Analysis of FL9 LPS

FL9 contains a mini-Tn5 transposon insertion in *pbpra2686*, a gene which encodes a protein with significant homology to tyrosine protein kinase, Wzc (Figure 53 and Table 18). In *E. coli*, the tyrosine protein kinase Wzc forms a part of a complex with Wza, Wzb, Wzx and Wzy. Wzc has been demonstrated to be required for the polymerisation and transport of CPS to the outer membrane in *E. coli* (Reid and Whitfield, 2005). A mutation in Wzc has been shown in *E. coli* to cause a loss of capsule and an increase in the expression of K_{LPS}. This section demonstrates that *pbpra2686* encodes a tyrosine protein kinase. This is achieved by comparing TEM images of the capsule and PAGE analysis of the LPS from FL9 to that of the parent strain.

5.3.1 The LPS profile of FL9 shows alterations that are characteristic of a *wzc* mutant

The LPS profile of the *P. profundum* SS9R mutant, FL9, was visualised by isolation of the LPS using the SDS-lysis method and separation by SDS-PAGE followed by alcian blue silver staining. The LPS profile of FL9 had a significant alteration in the intermediate bands compared to the parent strain (Figure 58). This has been previously shown to be a feature of Wzc mutants in *E. coli* (Wugeditsch *et al.*, 2001). Due to the fact that FL9 displayed cold temperature sensitivity only on marine agar and because the LPS of the parent strain was shown to alter when grown on marine agar (Chapter 4, Figure 29) the LPS from growth on marine agar and in marine broth were compared. When grown on marine agar, the LPS from FL9 had an alteration in the intermediate bands compared to the LPS when grown in marine broth (Figure 58, see arrow). At 15°C (0.1 MPa), the alterations in the LPS profiles of both the parent strain and FL9 had an additional uppermost band of the intermediate bands. When grown on agar at 4°C (0.1 MPa) the LPS profile of FL9 was similar to that seen on agar at 15°C (0.1 MPa), whilst the LPS from the parent strain grown under the same conditions was not. This suggests that *P. profundum* SS9R performs many modifications of the LPS depending

upon the growth conditions, and that FL9 did not perform the same modification after growth on marine agar at 4°C (0.1 MPa) that the parent strain did. This would indicate that *wzc* was required for those changes. Compositional analysis of either the whole LPS sample or the individual bands isolated from the PAGE would help to identify these LPS profile alterations present.

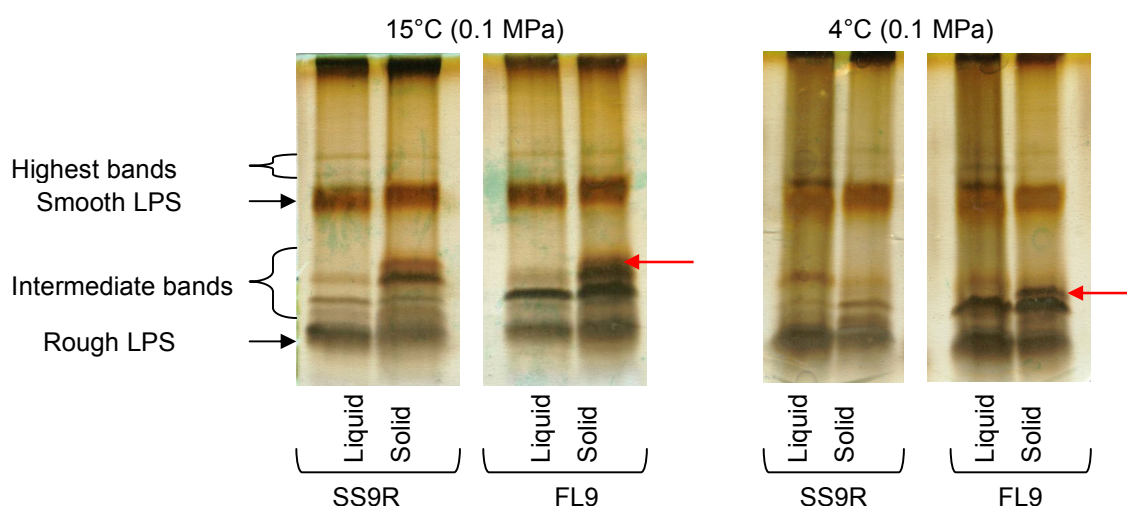


Figure 58: Disruption of the putative *wzc* affects the ability of *P. profundum* to adapt LPS profile in response to temperature and growth on agar

Cultures of *P. profundum* SS9R and FL9 were incubated at 15°C (0.1 MPa) and 4°C (0.1 MPa) in marine broth (anaerobic) and on marine agar plates. Stationary phase cultures from marine broth were pelleted by centrifugation. Colonies grown on marine agar plates were collected and resuspended in fresh marine broth before being pelleted. The LPS were isolated by SDS-lysis and then analysed by SDS-PAGE followed by alcian blue silver staining. Red arrow indicates LPS modifications.

Previous studies have shown that changing the method of polysaccharide extraction can reveal subtle differences between mutants. By using the hot phenol water method and DOC-PAGE the LPS profile can be analysed in greater detail and also the LPS can be purified for analytical purposes. A 1 litre culture was used for a hot phenol water extraction and the resulting LPS were analysed by DOC-PAGE. Using this method firstly allows the separation of hydrophilic and hydrophobic LPS. Since we have found that the majority of LPS molecules were isolated in the aqueous, or hydrophilic phase, the majority of work has been performed on the aqueous phase. The

gel shows the aqueous phase LPS from *P. profundum* SS9R and FL9 (Figure 59). FL9 has an increased intensity in an intermediate band compared to the parent strain (Figure 59A). Also, by using alcian blue staining, further alterations were detected (Figure 59B) which could correspond to the KLPS modification.

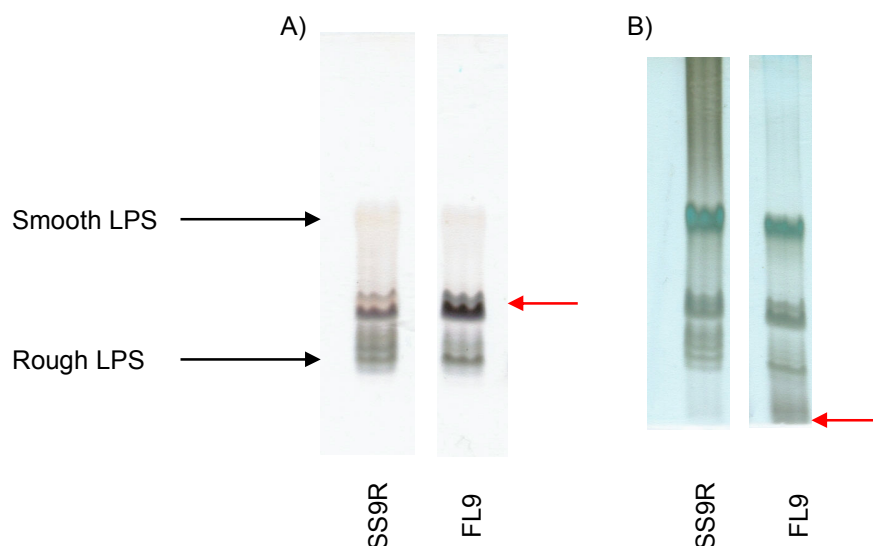


Figure 59: The DOC-PAGE of aqueous phase LPS profiles of *P. profundum* SS9R and FL9 reveal greater detail than the SDS-PAGE.

Stationary phase cultures from 15°C (0.1 MPa anaerobic) were diluted to a starting OD₆₀₀ 0.1 in 1 litre of fresh marine broth and were incubated at 15°C (0.1 MPa anaerobic). Once in stationary phase the cultures were pelleted by centrifugation and the LPS was isolated by the hot phenol water method. The aqueous phase LPS were then analysed by DOC-PAGE followed by either sodium-m-periodate silver staining (A) or alcian blue silver staining (B). Red arrow indicates LPS modifications.

5.3.2 FL9 does not have an alteration in biofilm or motility compared to the parent strain

Since the cold temperature sensitivity phenotype of FL9 was only apparent on solid marine agar (Figure 55 and Figure 56), the attachment of FL9 to a surface was compared to the parent strain. Cultures were incubated at 15°C (0.1 MPa aerobic shaking 200 rpm) in glass test tubes and the attachment was visually inspected. By this method the attachment of FL9 to the test tubes appeared similar to the parent strain (Figure 60).

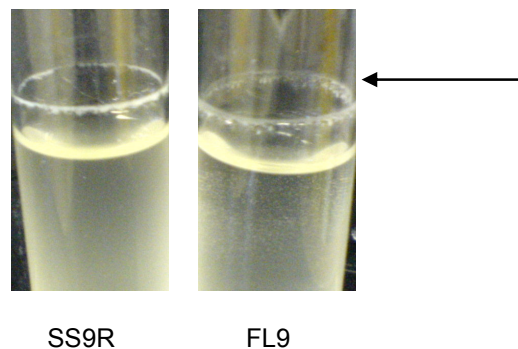


Figure 60: FL9 has a biofilm development similar to the parent strain

Anaerobic stationary phase cultures from 15°C (0.1 MPa) were diluted to an OD600 0.1 in fresh marine broth with 20 mM glucose and incubated at 15°C (0.1 MPa 200 rpm aerobic) for 48 hours.

As shown in Chapter 4 (Figure 32 and Figure 36), surface polysaccharide mutants can have alterations in motility. The motility of FL9 was therefore also assessed. FL9 had motility comparable to the parent at both 15 and at 4°C (Figure 61). These results therefore suggested that FL9 does not have an alteration in biofilm formation or motility compared to the parent strain.

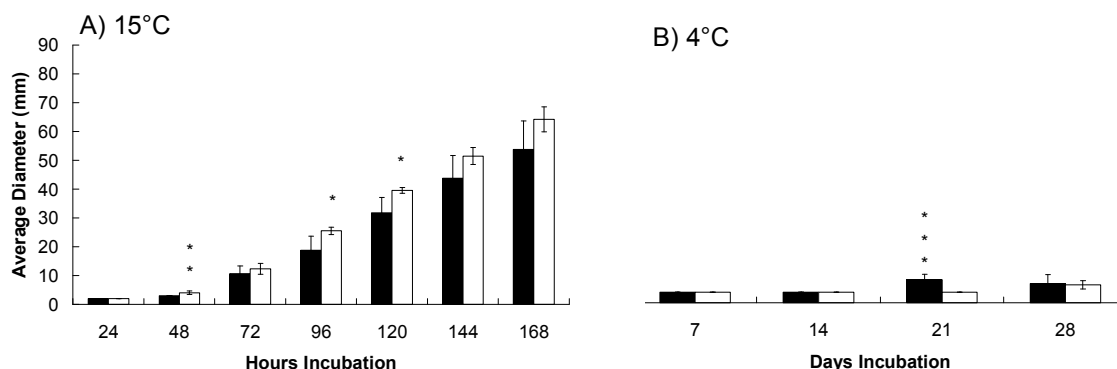


Figure 61: FL9 does not show significantly increased motility compared to *P. profundum* SS9R

Anaerobic stationary phase cultures from 15°C (0.1 MPa) were diluted to an OD₆₀₀ 1.0 in fresh marine broth and 3 µl was spotted onto the centre of a motility plate containing 0.3% w/v agar and 20 mM glucose. The plates were then incubated at 15°C and the diameter of measured each day. The average diameter was calculated from 3 motility plates with 2 diameter measurements each. *P. profundum* SS9R (filled), FL9 (white). The mean motility was compared with SS9R using the unpaired student's t-test, 2 tailed: P>0.05 not significant (no symbol), P<0.05 (*), P<0.01 (**), P<0.001 (***). Error bars represent ± 1 SD

5.3.3 FL9 has a reduction in capsule characteristic of Wzc mutants

To further investigate the effect of the transposon insertion in *pbpra2686* on FL9, the capsule was examined by TEM. Wzc mutants in *E. coli* have been demonstrated by TEM with cationised ferritin staining to have lost the capsule (Figure 54A) (Reid and Whitfield, 2005). Cationised ferritin is a charged stain that binds negatively charged residues, such as CPS.

P. profundum SS9R was shown to have a cationised ferritin staining surrounding most of the cell surface, which relates to the negatively charged CPS (Chapter 3.4, Figure 22) The mutant, FL9, was also found to have cationised ferritin staining when grown under anaerobic conditions, albeit at a reduced level compared to the parent strain (Figure 62). When FL9 was grown under aerobic conditions the cationised ferritin staining was almost completely lost. This indicated that FL9 has an alteration in capsule

expression that is similar to published *E. coli* Wzc mutants (Reid and Whitfield, 2005), which is also dependent on the growth conditions.

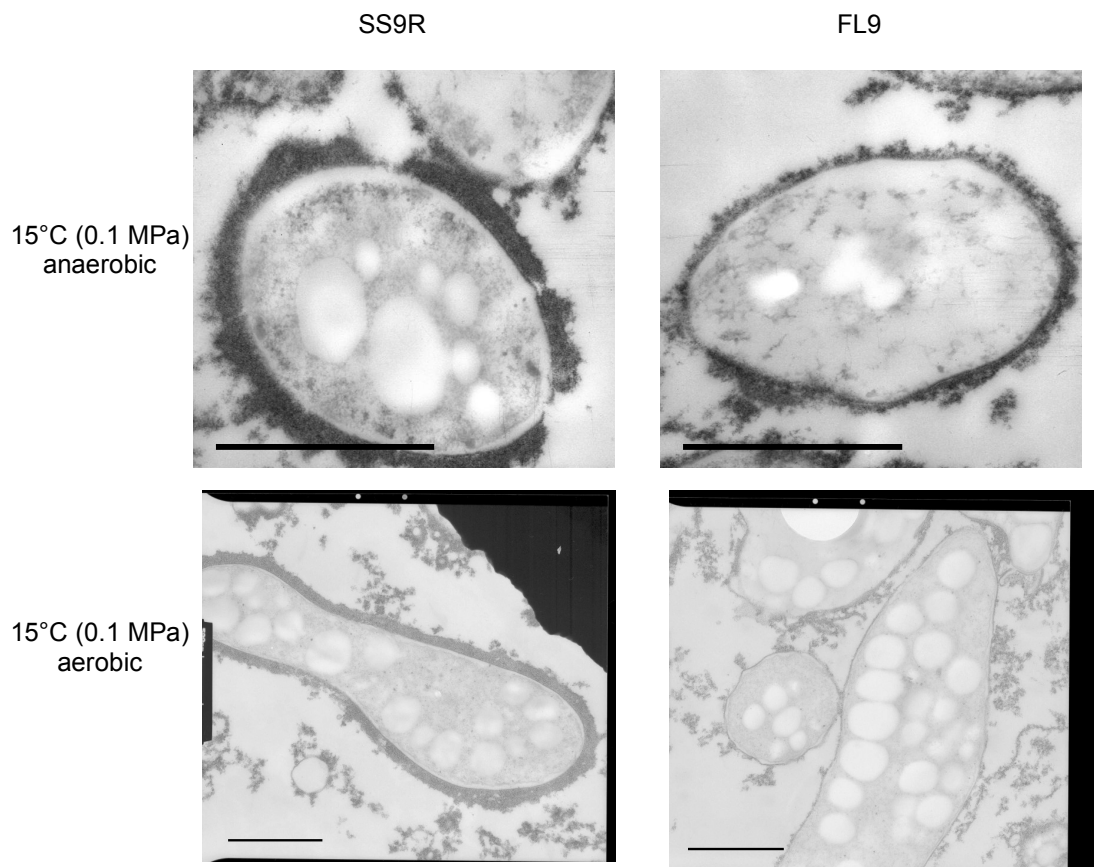


Figure 62: FL9 has a reduction in cationised ferritin staining compared to the parent

Aerobic and anaerobic stationary phase cultures of *P. profundum* SS9R and FL9 from 15°C (0.1 MPa) were prepared for TEM analysis with the addition of cationised ferritin (see method 2.8.4). Bar equals 1µm.

5.4 A comparison with the *Escherichia coli* *wzc* mutant, CWG285

The synthesis and transport of CPS in *E. coli* has been investigated for many years and is still on going (Whitfield, 2006). The *P. profundum* SS9 *wzc* mutant, FL9, was therefore compared to a characterised *wzc* mutant in *E. coli*, CWG285. This was aimed to find whether the cold temperature sensitive phenotype was shared by other *wzc* mutants.

5.4.1 A mutation in *wzc* in *E. coli* does not lead to cold temperature sensitive growth

As previously mentioned, the LPS profile of the *E. coli* *wzc* mutant has an increase in the intermediate bands which relates to the accumulation of KLPS (Figure 54B). By using the SDS-lysis method and SDS-PAGE with silver staining it was shown that the LPS alteration present in the *E. coli* *wzc* mutant was detectable by our methods (Figure 63A). In order to demonstrate whether the mutation in *wzc* would cause it to become cold temperature sensitive, the growth on LB agar plates at 37°C was compared to the growth at 15°C. At 37°C and 15°C both the parent and mutant strains had similar growth, to 10⁻⁴ dilution (Figure 63B), which showed that under these conditions the loss of CPS did not affect the cold temperature sensitivity on LB agar.

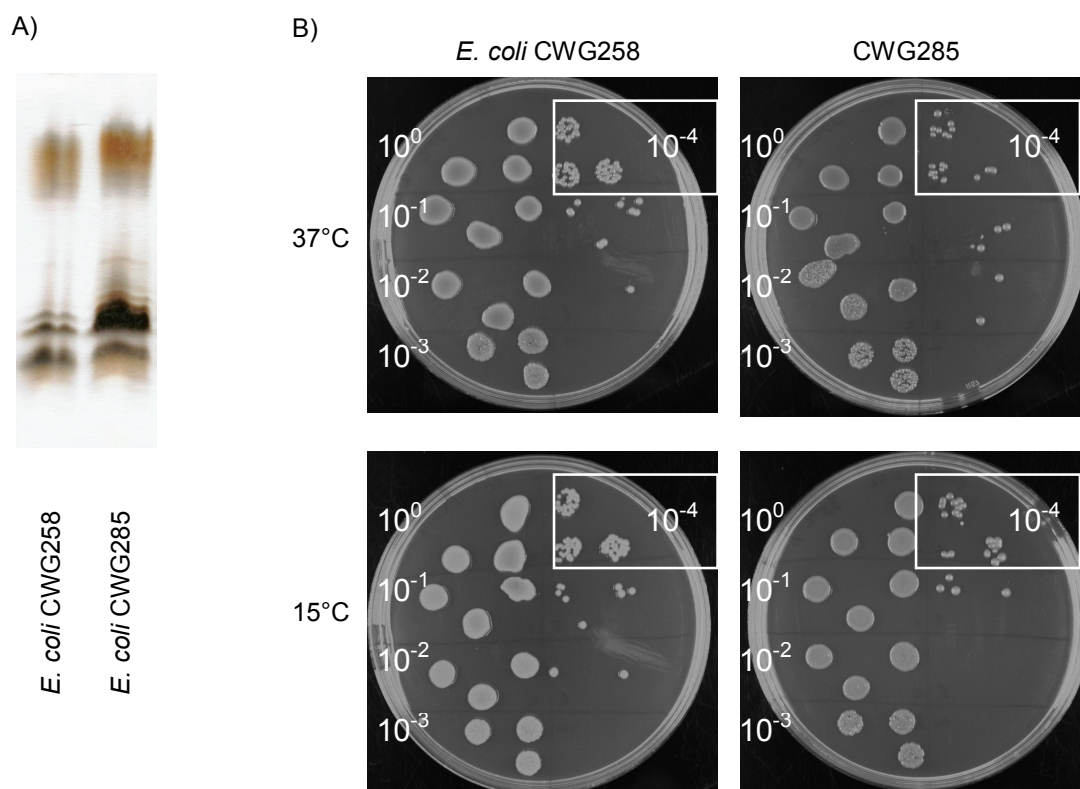


Figure 63: *E. coli* CWG258 and CWG285 do not display a cold temperature sensitive phenotype on LB agar

A) Stationary phase cultures of *E. coli* CWG258 and CWG285 (*wzc*-) from 37°C shaking culture were pelleted by centrifugation. The LPS were isolated by SDS-lysis and then analysed by SDS-PAGE followed by sodium-m-periodate silver staining.

B) Stationary phase cultures of *E. coli* CWG258 and CWG285 (*wzc*-) were diluted to an OD₆₀₀ 0.1 in fresh LB and serially diluted to 10⁻⁶. These dilutions were spotted in triplicates of 10 µl each onto marine agar plates. The plates were incubated at 37°C and 15°C. After 1 (37°C) and 4 days (15°C) the growth was assessed.

The growth of the *E. coli wzc* mutant and the parent strain at cold temperatures was also investigated in LB broth. At 37°C *E. coli* CWG285 had very similar growth to the parent strain (Figure 64A). The growth rate at 15°C was reduced compared to 37°C and both the parent and mutant strains showed very similar growth (Figure 64B). The *E. coli wzc* mutant therefore displayed no significant cold temperature sensitivity compared to the parent strain in LB broth at 15°C.

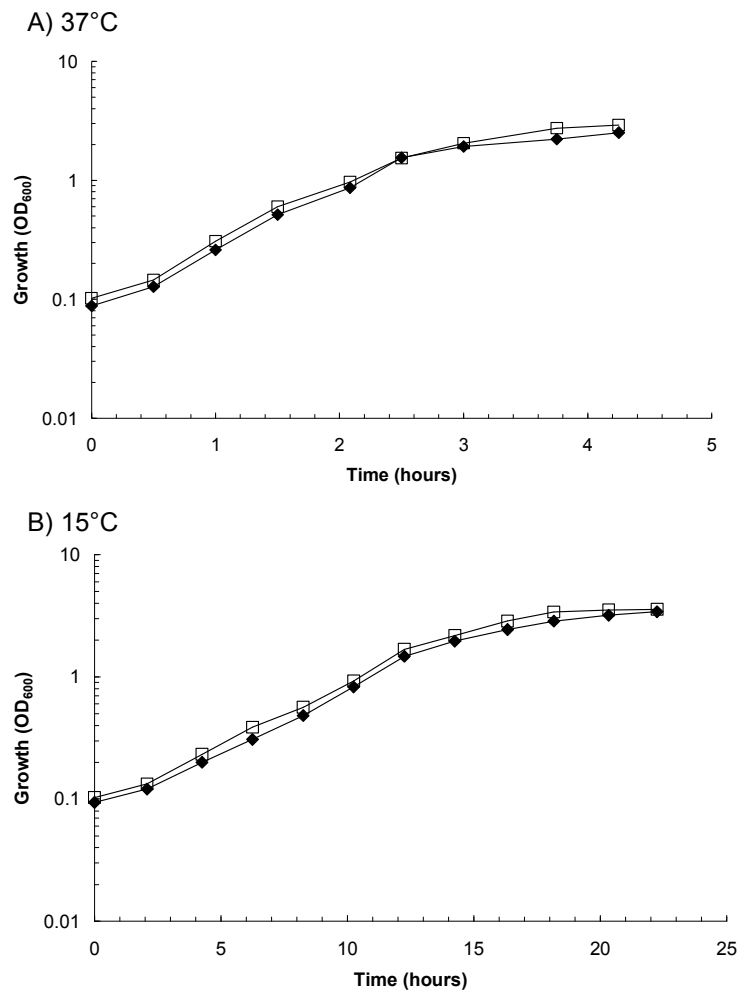


Figure 64: *E. coli* CWG285 mutant does not display a temperature sensitive phenotype in 15°C shaking liquid culture

Stationary phase cultures were diluted to an OD₆₀₀ 0.1 in fresh LB and incubated at 37°C (A) and 15°C (B), 200 rpm. The optical density was measured frequently as a measure of growth. *E. coli* CWG285 (♦) and CWG285 (*wzc*-) (□).

5.4.2 CWG285 has an increase in swimming motility compared to the parent strain

The motility of the *E. coli wzc* mutant was compared to the parent strain on 0.3% (w/v) LB agar. After 7 hours FL9 was shown to have moved 10 mm, whilst the parent strain had not yet moved significantly from the inoculation point (Figure 65). The *E. coli wzc* mutant continued to move significantly more than the parent strain, having moved 46 mm further than the parent strain at 23 hours. The motility alteration of the *E. coli wzc* mutant has not been shown before for.

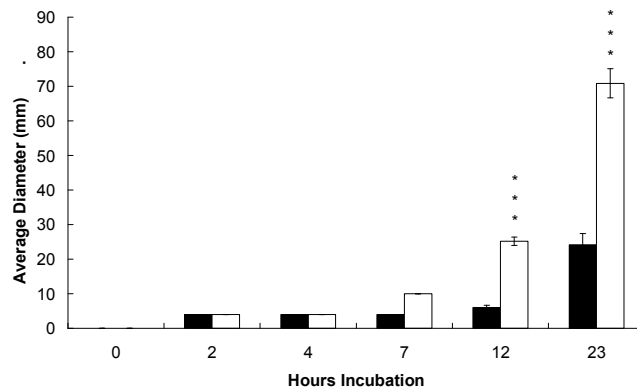


Figure 65: *E. coli* CWG285 has an increased motility compared to the parent strain

Stationary phase cultures were diluted to an OD₆₀₀ 1.0 in fresh LB and 3 µl was spotted onto the centre of an LB motility plate containing 0.3% w/v agar and streptomycin. The plates were then incubated at 37°C and the diameter of measured frequently. The average diameter was calculated from 3 motility plates with 2 diameter measurements each. *E. coli* CWG258 (filled) and CWG285 (*wzc*-) (white). The mean motility was compared with SS9R using the unpaired student's t-test, 2 tailed: P>0.05 not significant (no symbol), P<0.05 (*), P<0.01 (**), P<0.001 (***), untested (▲). Error bars represent ± 1 SD.

5.5 Complementation of *P. profundum* SS9R FL9

In order to demonstrate that the phenotypes observed in FL9 were due to the disrupted gene, *pbpra2686*, that gene must be complemented.

5.5.1 Expression of pDA02 (pFL190 *pbpra2686*) will partially complement the LPS profile alteration in FL9

To confirm that the phenotypes seen in FL9 were due to the mini-Tn5 transposon insertion in *pbpra2686*, complementation experiments were performed. *pbpra2686* was amplified by PCR, digested with *Bsa*I and *Xba*I and cloned into pFL190 (see Appendix and methods 2.10 for cloning procedures). The new construct was then transformed into *E. coli* DH5 α and screened by restriction digest. The successful clone was sequenced, to confirm that the gene sequence was correct and that the promoter region on pFL190 was also intact. The new construct, now pDA02, was then mated into FL9 using the *E. coli* helper strain CSH56 with pRK2013.

Firstly, the ability of pDA02 to complement the cold temperature sensitive phenotype of FL9 was tested. Growth of FL9 and the parent strain (both with pFL190) were very similar at 15°C on marine agar plates containing glucose, with both strains growing to 10⁻⁴ dilution (Figure 66). FL9 pFL190 and FL9 pDA02 both grew to 10⁻⁴ dilution at 4°C on marine agar plates with glucose. Marine agar plates containing 0.1% (w/v) arabinose at 15°C also grew to 10⁻⁴ dilution, although FL9 pDA02 had more growth at this dilution than FL9 pFL190. The largest difference came when plates marine agar plates containing 0.1% (w/v) arabinose were incubated at 4°C. Under this condition all growth was reduced. The parent strain with pFL190 had growth until 10⁻³ dilution whereas FL9 pFL190 had little noticeable growth. FL9 pDA02 had growth at 10⁻² dilution, although this was large mucoid colonies.

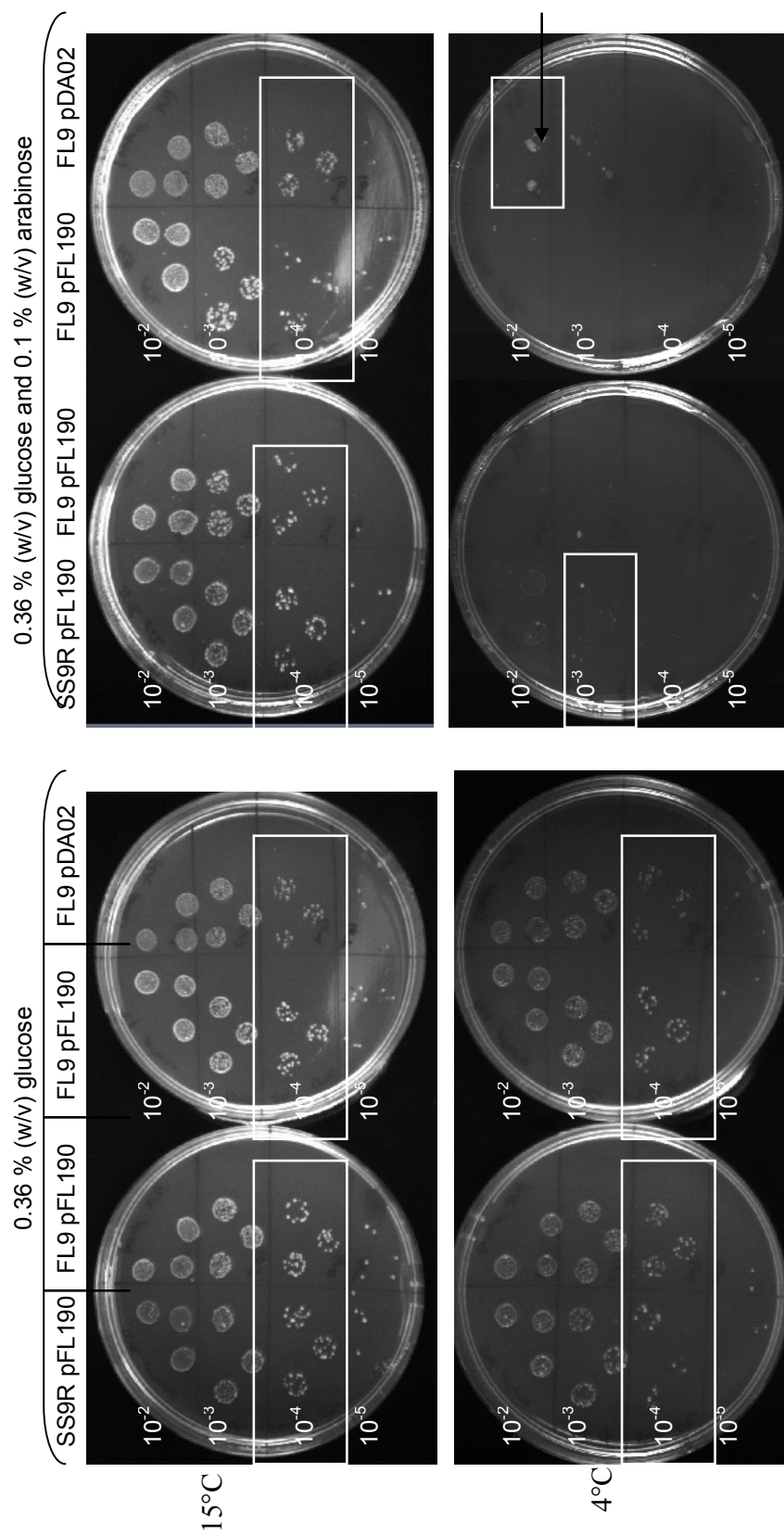


Figure 66: Cold temperature sensitive phenotype is masked by growth on streptomycin

Stationary phase cultures from 15°C (0.1 MPa shaking 200 rpm aerobic) liquid cultures were diluted to an OD_{600} 0.2 in fresh marine broth and serially diluted to 10^{-5} . These dilutions were spotted in triplicates of $10 \mu\text{l}$ each onto marine agar plates with $0.36\% \text{ (w/v)}$ glucose, and arabinose where shown and streptomycin. Plates were incubated for 5 days for all plates at 15°C , 14 days for marine agar plates with glucose at 4°C and 31 days for marine agar plates with arabinose at 4°C . Plates incubated with $1\% \text{ (w/v)}$ arabinose grew poorly and are not shown. Arrow indicates mucoid colonies growth. White boxes highlight the lowest dilution with significant growth in all 3 spots.

Secondly, the ability of pDA02 to complement the LPS alteration phenotype in FL9 was assessed. Cultures were grown in aerobic shaking culture with additional arabinose. The LPS was isolated by SDS-lysis and analysed by SDS-PAGE. In these gels sodium-m-periodate silver staining was used in place of alcian blue silver staining as alcian blue was found to stain too heavily to detect the subtle changes in LPS profile that arose from the complementation. An initial gel had a possible reduction in intensity of the intermediate band alteration seen in FL9, however it was difficult to distinguish (Figure 67A). By further diluting the samples it was made clear that expression of pDA02 was affecting the LPS profile of FL9, making it more like the parent profile (Figure 67B).

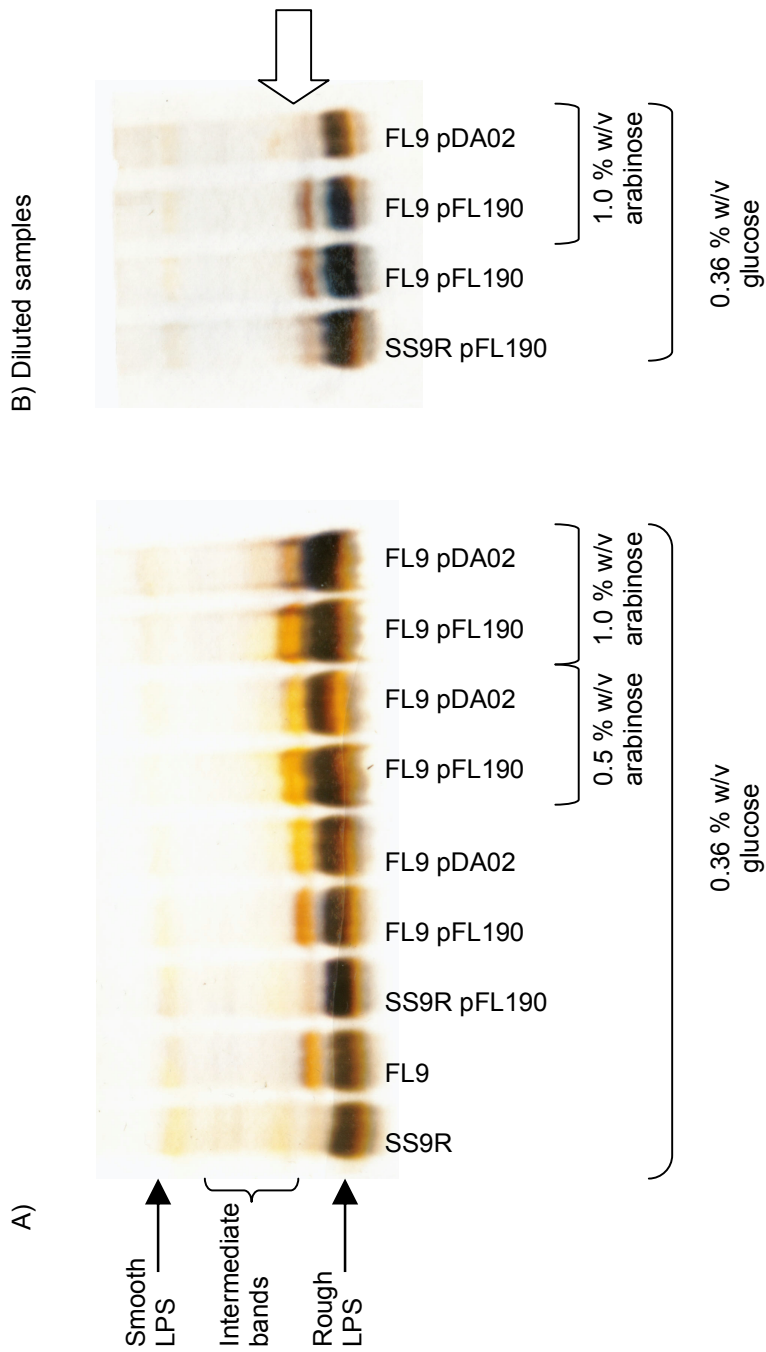


Figure 67: Expression of *pbpra2686* (pDA02) will partially complement the LPS SDS-PAGE profile of FL9

Stationary phase cultures from 15°C (0.1 MPa shaking 200 rpm aerobic) liquid cultures were pelleted by centrifugation. The LPS were isolated by SDS-lysis and then analysed by SDS-PAGE followed by sodium-m-periodate silver staining. *P. profundum* SS9R, SS9R pFL190, FL9, FL9 pFL190 and FL9 pDA02 were grown in the presence of 0.36% w/v glucose and FL9 pDA01 in 0.5% and 1.0% w/v arabinose. A) Initial investigation of LPS complementation and B) after sample dilution. White arrow indicates partial complementation of mutant LPS phenotype.

5.6 Discussion

5.6.1 The *P. profundum* SS9 gene, *pbpra2686*, encodes a tyrosine protein kinase, Wzc

FL9 is a mutant strain of *P. profundum* SS9R that contains a mini-Tn5 transposon insertion in *pbpra2686*, a gene annotated as a putative tyrosine protein kinase. This chapter demonstrated by SDS-PAGE that the LPS profile of FL9 shares similarities with the *E. coli* *wzc* mutant, CWG285. The alteration in the *E. coli* *wzc* mutant is an increase in intermediate band intensity which has been shown to be due to a build up of KLPS (Drummelsmith and Whitfield, 1999; Wugeditsch et al., 2001). This is a unique feature of *E. coli* that produce a group 1 capsule. FL9 also had an increase in intermediate band intensity which was remarkably similar to the *E. coli* *wzc* mutant. *pbpra2686* encodes a protein with 58% identity to a Wzc from *Vibrio vulnificus* and 67% similar to *E. coli* K12, therefore suggesting that *pbpra2686* does encode for Wzc.

The LPS profile of FL9 did not significantly change in response to temperature, as previously seen with *P. profundum* SS9R. However, due to the fact that the mutants only displayed cold temperature sensitivity on marine agar the LPS molecules were isolated from marine agar instead of marine broth. This demonstrated that FL9 exhibited a further LPS profile alteration when grown on marine agar and that at 15°C *P. profundum* SS9R and FL9 had a similar alteration, gaining another uppermost intermediate band. At 4°C however, the parent LPS profile looked markedly different to that from 15°C. The FL9 4°C LPS profile from agar looked very similar to that seen at 15°C. This indicated that the parent strain modifies the LPS in response to growth conditions, such as liquid or solid media, and temperature. Due to the fact that FL9 appeared to perform the same modifications at 15 and 4°C on agar, it is reasonable to assume that *pbpra2686* is required for certain LPS modifications. Alternatively, the KLPS addition previously mentioned might inhibit the normal LPS modifications in some way.

The major feature of a *wzc* mutation in *E. coli* is the loss of the high molecular weight capsule, which is most commonly analysed by western blotting or TEM with cationised ferritin staining. This study showed that FL9 also shared this phenotype. Interestingly, through the course of the investigation it was found that the degree of capsule loss varied depending on growth conditions. Specifically, FL9 grown aerobically had a complete loss of the capsule, whereas when grown anaerobically only had a partial loss. This may be explained by the fact that the expression of extracellular polysaccharide such as CPS and EPS are known to be affected by growth conditions, such as repression of *E. coli* K1 sialic acid capsule at cold temperatures (Grant *et al.*, 1969; Merker and Troy, 1990).

Expression of pDA02 (pFL190 with *pbpra2686*) with 0.5 and 1.0% (w/v) arabinose was shown to affect the LPS profile of FL9, by reducing the relative intensity of the intermediate band alteration of this mutant. By using dilutions of the LPS samples it was shown that LPS from FL9 pDA02 grown in the presence of 1% (w/v) arabinose was very similar to the parent LPS. Unfortunately, whether expression of pDA02 in FL9 will affect the capsule alteration has not yet been investigated.

5.6.2 FL9 displays a cold temperature sensitive phenotype on marine agar

FL9 has been shown to have a cold temperature sensitive growth phenotype that is only apparent when grown on marine agar and not in liquid. This was found to be a reproducible 10 fold reduction in growth compared to the parent strain at 4°C (0.1 MPa). Because FL9 showed no significant alteration in growth at 15°C on marine agar or in marine broth, the phenotype was not a general growth defect.

It has been postulated that EPS and CPS are involved in low temperature adaptation (Corsaro *et al.*, 2004). It is therefore possible that the capsule forms a protective layer, and that this has more of an effect when grown on solid media (immobilised *E. coli* K12 have a different protein expression response to cold shock than

suspended cells (Frédérique Perrot, 2001)). The LPS alteration may also have an effect on the stability of the outer membrane and, consequently, cold temperature sensitivity.

pFL190 is under the control of an arabinose inducible promoter, therefore arabinose was required for complementation studies. Streptomycin was also included for plasmid maintenance. At 15°C (0.1 MPa), all cultures with/without arabinose had grown comparably after 5 days incubation. However, experiments at 4°C (0.1 MPa) which included arabinose significantly affected the growth of *P. profundum* SS9 strains, which resulted in the loss of the original cold temperature sensitivity phenotype. At 4°C, marine agar plates which lacked arabinose had significant growth after 14 days. Marine agar plates at 4°C which contained arabinose took 31 days to see significant growth, 16 days longer than those without arabinose. The growth on marine agar plates with arabinose at 4°C was also significantly reduced compared to the same plates at 15°C. It would seem that the combination of streptomycin, arabinose and cold temperatures is detrimental to *P. profundum* SS9 growth. Even so, FL9 pDA02 did have growth of some large mucoid colonies that was not seen on FL9 pFL190.

5.6.3 The *E. coli* *wzc* mutant, CWG285, was found to have an increased motility

In this chapter, it was discovered that the *E. coli* *wzc* mutant, CWG285, had a significantly increased motility compared to the parent strain. To our knowledge, this is the first finding of a motility alteration resulting from a loss of capsule in *E. coli*. However, bacterial tyrosine phosphorylation systems are diverse and have an expanding and diverse range of effects on virulence, DNA metabolism, sporulation and motility (Grangeasse *et al.*, 2007; LaPointe *et al.*, 2008). A tyrosine kinase found in *Myxococcus xanthus* has been shown to affect pilli-mediated motility through interaction with a GTPase (Bobbie Thomasson, 2002). It is therefore possible that the *E. coli* Wzc is having an effect on signal transduction through phosphorylation that has not yet been documented and therefore elucidated. Loss of the capsule could equally likely increase

motility by affecting the “wetability” of the cells and by exposing cell structures such as pili, that are hidden by the capsule.

5.6.4 Summary and conclusions

The phenotypes arising from the transposon insertion in the *wzc* of *P. profundum* SS9R, *pbpra2686*, can be explained by direct effects on the LPS structure, the KLPS modification seen in the *E. coli wzc* mutant, CWG285, may affect the stability of the membrane and therefore it's stability under temperature stresses. This could also be a result of the loss/reduction in capsule, which has been postulated to have an influence on adaptation cold temperature. However, due to the mutation in *pbpra2686* having such diverse effects on the cell envelope it is difficult to isolate which alteration is responsible for the phenotype or whether it is a combination of the two. To further investigate the underlying mechanism it would be useful to construct a mutant in a protein which would have no effect on LPS structure. In *E. coli* group 1 capsules Wzy is responsible for the polymerisation of capsule subunits. A mutation in *wzy* would create a loss of capsule without having the build up of KLPS. Unfortunately, while *P. profundum* SS9 shares significant homology with the *E. coli* group 1 capsule system, there is no significant homolog to Wzy. *E. coli* group 2 and 3 capsules are polymerised by a glycosyltransferase activity (Whitfield, 2006). *pbpra2685* is immediately downstream of *pbpra2686* and shares significant homology with a glycosyltransferase (80% similarity) and would therefore be in ideal target. There is also the possibility that *P. profundum* SS9 uses a system similar to the *E. coli* group 4, having homologues to Wza, Wzb and Wzc, but also requiring WecA for the initiation of capsular subunit synthesis. *P. profundum* SS9 contains a gene with significant homology to WecA, it is likely that *P. profundum* SS9 utilises a system similar to the *E. coli* group 4 capsular expression system. Therefore, a mutant in WecA would also prove valuable in determining the role of the capsule in cold temperature adaptation. Also, the expression of the capsule under various environmental conditions would be useful in determining it's effect on the survival of *P. profundum* SS9 under extreme conditions.

6. Concluding remarks

Photobacterium profundum SS9 is a piezophilic and psychrophilic bacterium that is capable of growing over a range of pressures (0.1–90 MPa) and temperatures (2–20°C) and grows optimally at 28 MPa 15°C (DeLong, 1986). In order to investigate the mechanisms of high pressure and cold temperature adaptation in *P. profundum* SS9, the growth at cold temperatures and high pressures was first described in detail. This involved growth curves at 0.1 and 28 MPa (15°C) and the assessment of growth on marine agar at 2, 9 and 15°C (0.1 MPa) in order to gain an understanding of the growth of *P. profundum* SS9 that has not been previously covered in significant detail.

Apart from the growth experiments, the surface polysaccharides of *P. profundum* SS9 grown under different conditions were also investigated. The unsaturated fatty acid content of *P. profundum* SS9 outer membrane has been previously shown to increase in response to growth at high pressure and cold temperatures and that C18:1 is required for growth at high pressures (Allen *et al.*, 1999; Allen and Bartlett, 2000). By hot phenol water isolation and DOC-PAGE, it was demonstrated that the surface polysaccharide profile changed at 0.1 MPa with respect to temperature but not at 28 MPa. These alterations were shown not to occur in the piezo-sensitive *P. profundum* 3TCK or *S. Typhimurium* LT2. Alterations in LPS composition have been demonstrated to occur in many bacteria in response to growth temperature (Corsaro *et al.*, 2001; Cronan, 1968; Knirel *et al.*, 2005; Kumar *et al.*, 2002; Marr and Ingraham, 1962; Ray *et al.*, 1994; Sinensky, 1971). However, these are usually changes in fatty acid composition and, to our knowledge, a change in the LPS in response to temperature has not been demonstrated by DOC-PAGE before. This could indicate that the polysaccharide alterations occurring in *P. profundum* SS9 are somehow unique.

To further investigate the changes in polysaccharide profile in *P. profundum* SS9, the polysaccharides were purified and analysed for carbohydrate and fatty acid content by GC-MS. Unfortunately, these experiments showed that the carbohydrates consisted mostly of glucose (at least 99 %). Due to the large amount of glucose present, it appears that the major polysaccharide of *P. profundum* SS9 is a glucan. Later

experiments demonstrated that by growing the *P. profundum* SS9 cultures aerobically instead of anaerobically the overall glucose content was reduced, which allowed the detection of a number of other carbohydrates. This will hopefully allow further compositional analysis of *P. profundum* LPS and allow for the identification of the modifications identified by DOC-PAGE.

Two previously isolated *P. profundum* SS9R mini-Tn5 transposon mutants, FL26 and FL9, had been characterised as cold temperature sensitive on marine agar only at 4°C 0.1 MPa compared to the parent strain (Lauro *et al.*, 2008). These mutants contained transposon insertions in genes encoding a putative O-antigen ligase and a putative tyrosine protein kinase (Wzc), respectfully. This study has shown that FL26 has an LPS alteration similar to previously published O-antigen ligase mutants in bacteria such as *P. aeruginosa*, *V. cholerae* and *E. coli* (Abeyrathne *et al.*, 2005; Heinrichs *et al.*, 1998a; Schild *et al.*, 2005). Interestingly, FL26 and a number of other putative polysaccharide biosynthesis mutants (2 glycosyltransferase mutants and a putative Wzx) were shown to have increased motility compared to the parent strain on 0.3 % (w/v) marine agar. Due to the agar concentration used, it was most likely that this related to swimming motility and the flagella. Due to the fact that the *P. profundum* SS9 genome encodes 2 flagella systems (Vezzi *et al.*, 2005) and that the expression of these has been shown to be dependant of viscosity and pressure (Eloe *et al.*, 2008), the flagella of FL26 was investigated. It was postulated that the LPS mutants were expressing the high pressure flagella system and that this led to the increased motility. By using a flagellar stain and a fluorescent protein stain it was found that neither FL26 nor the other affected mutants had a significant alteration in flagella structure. With the exception of FL9, the putative polysaccharide biosynthesis mutants were also shown to have altered biofilm formation phenotypes on glass test tubes compared to the parent strain. This included a range of phenotypes, such as significantly increased binding, significantly reduced binding, binding in different zones or retarded binding compared to the parent strain. Ideally this could be more accurately quantified using crystal violet staining. The crystal violet stains bound bacteria, which after solubilisation with acetic acid can later be measured using a spectrophotometer.

Since motility and biofilm formation have been linked in previous studies (Stafford and Hughes, 2007), it is therefore likely that the 2 phenotypes are related. Motility has been previously shown to be negatively affected by LPS alterations (Toguchi *et al.*, 2000) and this may simply be explained by an alteration in surface wettability, i.e. the surface alterations allow mutants to move more easily through the medium and such alterations also alter the biofilm formation. LPS alterations have also been demonstrated to affect the protein content of outer membranes (Ames *et al.*, 1974). This could also affect other cellular mechanisms and therefore an alternative explanation is that alterations in the composition of the outer membrane have an effect on nutrient uptake or signalling within the cell which ultimately affect motility.

It is therefore proposed that alterations in the LPS composition affect the cold temperature sensitivity of *P. profundum* SS9 and that these alterations have secondary phenotypes that are either directly or indirectly related. The effect of LPS composition on cold temperature sensitivity can be explained by alterations in membrane viscosity. Many bacteria have been shown to alter membrane composition in response to temperature known as homeoviscous adaptation (Sinensky, 1974). *P. profundum* SS9 has been shown to alter fatty acid composition in response to growth temperature and pressure and C18:1 was shown to be required for growth at high pressures (Allen *et al.*, 1999; Allen and Bartlett, 2000). Membrane fluidity affects many processes, such as nutrient uptake and diffusion (Nikaido and Vaara, 1985) and therefore an incorrect membrane composition, in this case due to the LPS alteration, has a harmful affect on the cell.

The putative tyrosine protein kinase mutant, FL9, also exhibited an LPS alteration and a reduction in CPS expression similar to previously published mutants in *wzc* in *E. coli* (Reid and Whitfield, 2005; Wugeditsch *et al.*, 2001). This suggested that alterations in the LPS and CPS can affect the cold temperature sensitivity of *P. profundum* SS9. While alterations in the LPS have been previously demonstrated to affect sensitivity to certain cell envelope stresses (Bennett *et al.*, 1981; Thomsen *et al.*, 2003), EPS has also been shown to protect against desiccation and freeze-thaw damage (Ophir and Gutnick, 1994; Tamaru *et al.*, 2005). The CPS may therefore affect cold

temperature sensitivity in 1 of 2 ways: 1) by the same mechanism postulated for the LPS mutants because Wzc mutations can result in the modification of LPS with CPS subunits, making KLPS (Cox and Perry, 1996; Drummelsmith and Whitfield, 1999; Wugeditsch *et al.*, 2001); or 2) due to the fact that EPS has been shown to be important for survival of desiccation and freeze-thaw damage (Ophir and Gutnick, 1994; Tamaru *et al.*, 2005), the CPS and EPS may form a moisture layer that protects against cold temperatures. Indeed *P. profundum* SS9 was shown to express a thicker capsule at 4°C compared to 15°C (0.1 MPa). It is therefore difficult to form a conclusion about the cause for cold temperature sensitivity in FL9. In order to discriminate between the CPS and LPS, a mutant that only has a defined effect on the CPS expression must be examined.

Additional work performed into the psychrophilic and piezophilic adaptations of *P. profundum* SS9 included the investigation in the cold/high pressure adaptation individual of proteins. As previously described, individual proteins have been previously shown to be adapted to cold temperatures in other bacteria (Langridge and Morita, 1966; Ryu *et al.*, 2005). This work concentrated on the Lactate dehydrogenase (Ldh) because dehydrogenases such as Ldh and Mdh have been used to examine protein biochemical adaptations to extreme environments (Welch and Bartlett, 1997). While the gene (*pbpra1210*) was successfully cloned, there were several issues involved in expressing the protein in a soluble, and therefore useful, form. Eventually the Ldh was solubilised using 8 M Urea, however there was insufficient time remaining in the project for characterisation of the purified protein and its activity at high pressures or cold temperatures.

Further work into the role of LPS alterations in the cold temperature sensitivity, motility and biofilm phenotypes of *P. profundum* SS9 should include a study of outer membrane protein composition and investigate any possible affects on chemotaxis.

Appendix A – Cloning of *P. profundum* SS9 O-antigen ligase, *pbpra0218*

In order to demonstrate that the phenotype seen was due to the disrupted gene, that gene must be complemented. In this section it was necessary to clone the disrupted gene onto a vector designed for use with *P. profundum* SS9 (pFL190) and which has been previously shown to function in *P. profundum* SS9 (Lauro *et al.*, 2005). Firstly the gene of interest was amplified by PCR, in this case the *waaL* gene, *pbpra0218* (predicted 1180 bp). This had to include the ribosomal binding site (RBS) as pFL190 only includes the promoter sequence. To find the optimum annealing temperature a gradient was used, finding the optimum temperature to be 61.8°C which gave the strongest amplification (Figure 68). The PCR product was then cleaned up and used for subsequent restriction digests.

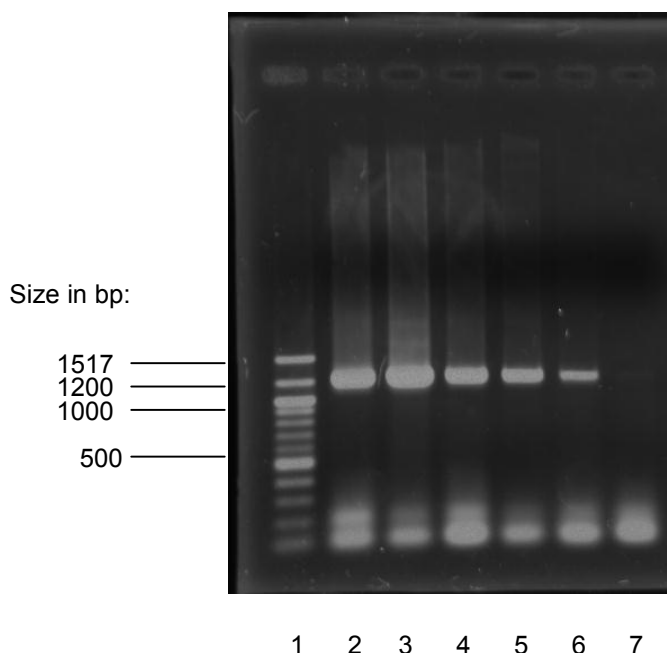


Figure 68: Temperature gradient PCR of *pbpra0218*

The gene *pbpra0218* was amplified using the primers Pbpra0218-F_*EcoRI* and Pbpra0218-R_*XbaI* by PCR using a temperature gradient to find the optimum temperature, separated on 0.8% w/v agarose gel. 100 bp DNA ladder (NEB) (1), and the following annealing temperatures: 60.5°C (2), 61.8°C (3), 63.1°C (4), 64.2°C (5), 65°C (6), -ve control no template DNA, 64°C (7). 1 minute 30 seconds extension time.

The *pbpra0218* PCR product was digested using *XbaI* and *EcoRI* (Figure 69, lane 8) and pFL190 was also digested (Figure 69, lane 5). The products were then used in a ligation of the double digested gene and pFL190. After overnight ligation at 4°C the ligation mix was added to 25 μ l competent cells from NEB and a basic transformation procedure was followed.

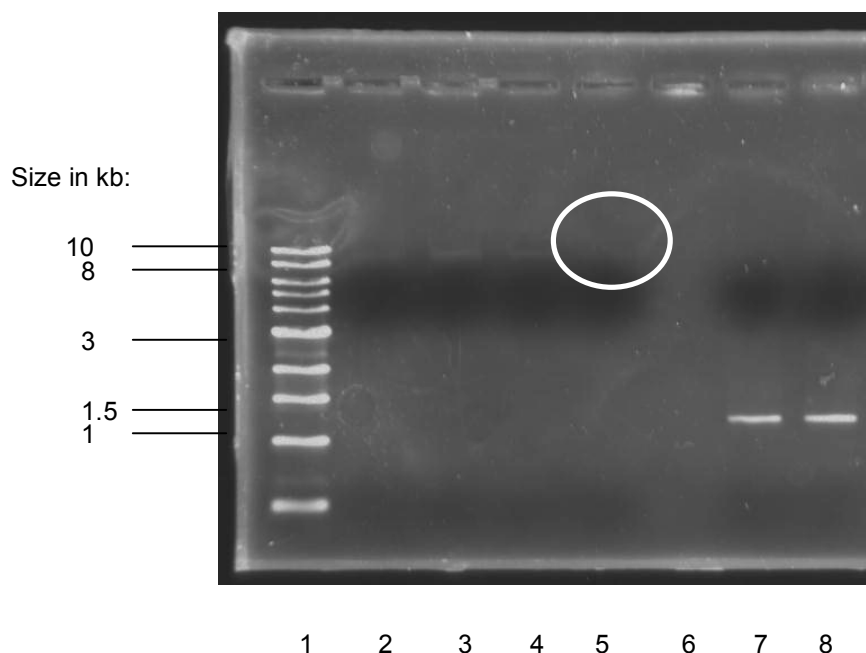


Figure 69: Restriction digest of pFL190 and *pbpra0218* PCR

Both plasmid and PCR were double digested using *XbaI* and *EcoRI* and separated on 0.8% w/v agarose gel. 1 Kb DNA ladder (NEB) (1), pFL190 (2), pFL190 *XbaI* digested (3), pFL190 *EcoRI* digested (4), pFL190 double digested (5), empty (6), *pbpra0218* PCR (7), *pbpra0218* PCR double digested (8). White circle highlights pFL190 double digested band.

Transformants were isolated and purified on LB agar with 100 $\mu\text{g.ml}^{-1}$ streptomycin before being screened by restriction digest. For example, the plasmids from clones 21-27 were extracted by QIAprep minispin kit. The plasmids were then digested using *XbaI* and *EcoRI* and the digested and undigested plasmids were visualised by agarose gel (Figure 70). Lanes 8 and 9 show that clone 24 contained an insert. Even though an excised band of the correct size (~ 1000 bp) could not be seen, both the supercoiled uncut band and the cut bands were shifted up compared to the other clones, which indicated that the plasmid contained something that the others did not.

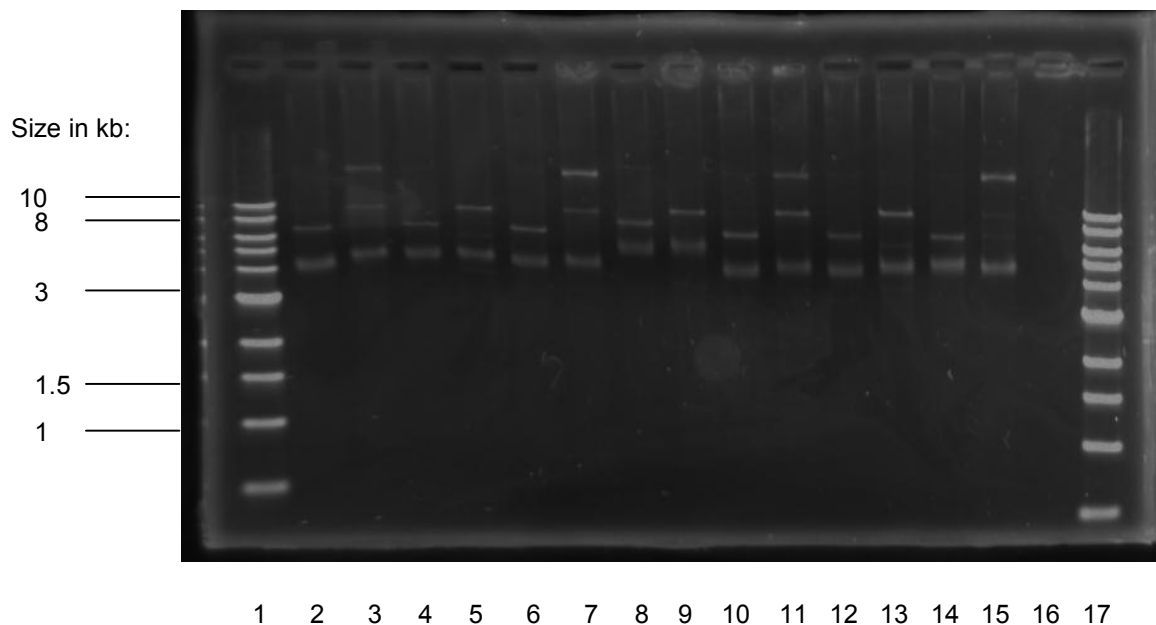


Figure 70: Screening *E. coli* DH5 α pFL190+*pbpra0218* clones by restriction digest

Following ligation and transformation, those clones growing on LB with streptomycin were purified and the plasmids extracted. The plasmids were then digested with *Xba*I and *Eco*RI and separated on 0.8% w/v agarose gel. 1 Kb DNA ladder (NEB) (lanes 1 and 17), clones 21-27 (lanes 2, 4, 6, 8, 10, 12 and 14) double digested (lanes 3, 5, 7, 9, 11, 13 and 15).

The digest of clone 24 was therefore repeated, this time with more starting DNA and more digested sample was loaded onto the gel (Figure 71). This shows a band of approximately 9.5 kb (pFL190), a supercoiled band running at 6-7 bp and a band between 1 and 1.5 kb, highlighted by the white circle (*pbpra0218* insert).

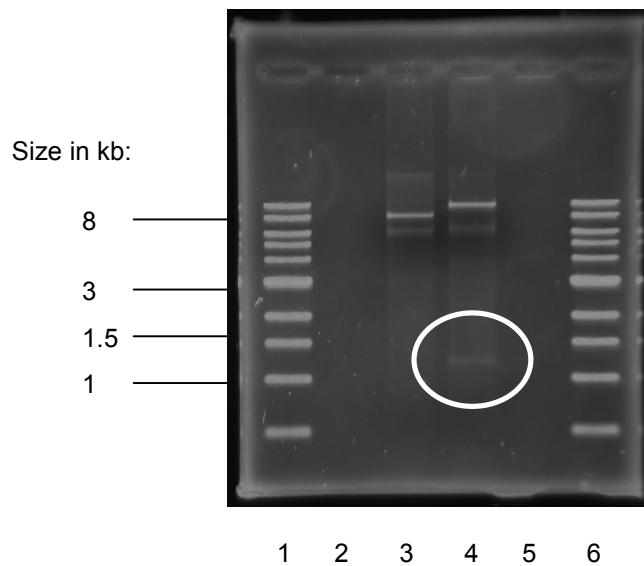


Figure 71: Restriction digest of clone 24

Those clones growing on LB with streptomycin were purified and the plasmids extracted. The plasmid were then digested with *XbaI* and *EcoRI* and separated on 0.8% w/v agarose gel. 1 Kb DNA ladder (NEB) (lanes 1 and 6), clone 24 (3), clone 24 double digested (4). White circle highlights *pbpra0218* band.

The plasmid was sequenced using pFL190 sequencing primers (pFL190-F and pFL190-R) to ensure accurate data for the start and finish of the gene, and the promoter sequence of the plasmid (Figure 72 and Figure 73). The sequence was found to be accurate and the plasmid was then renamed pDA01. pDA01 was then mated in *P. profundum* SS9R and FL26 using the mating protocol outlined in methods 2.10.11.

pDA01_F GGGTAAGTGTCTATATCACGGCAGAAAGTCCACATTGATTATTTGCACGGCGTCACACTT
 pbpra0218_F -----

pDA01_F TGCTATGCCATAGCATTATTTTATCCATAAGATTAGCGGATCCTACCTGACGCCTTTTATCG
 pbpra0218_F -----

pDA01_F CAACCTCTCTACTGTTTCTCCATAACCCGTTTTTTTGGGCTAGCGAATTCAGAGAATATTTT
 pbpra0218_F -----

pDA01_F TAAACTACTGATGTTATTGCCCTATATCTGGCTTTCGACCGGATTATTAGTGCTACGTAA
 pbpra0218_F TAAACTACTGATGTTATTGCCCTATATCTGGCTTTCGACCGGATTATTAGTGCTACGTAA

pDA01_F TAGCGACAAAACCATGATCGTCATGATGGTCATAGCCATTGCTGCCACCCTGTTCCATCG
 pbpra0218_F TAGCGACAAAACCATGATCGTCATGATGGTCATAGCCATTGCTGCCACCCTGTTCCATCG

pDA01_F CGGATTGGCTCCGATAAAAAGAAAACCTTAAAAAGGACAAAACCTTATGGGTACTATTGGC
 pbpra0218_F CGGATTGGCTCCGATAAAAAGAAAACCTTAAAAAGGACAAAACCTTATGGGTACTATTGGC

pDA01_F AATTACTGGCTATGCATTATTTGCTTATTTTATCATGGCGGTAGCTCACGAGAAATTCG
 pbpra0218_F AATTACTGGCTATGCATTATTTGCTTATTTTATCATGGCGGTAGCTCACGAGAAATTCG

pDA01_F TGCCTTGATTGGTGCATCCTTATTTTAAATTTGTTTTCCGAGAGAGCTGCTAACTCAAAG
 pbpra0218_F TGCCTTGATTGGTGCATCCTTATTTTAAATTTGTTTTCCGAGAGAGCTGCTAACTCAAAG

pDA01_F AATCTTATTAGGGTTAACTACTCTTAGCATCATCTCTCTGCTTAGCATATGCGTGTTACTA
 pbpra0218_F AATCTTATTAGGGTTAACTACTCTTAGCATCATCTCTCTGCTTAGCATATGCGTGTTACTA

pDA01_F TAATTTATATCTTAATATAGGTCGTAGTAGATGGCCAATCAATGCAATCCCTTTCGCAAC
 pbpra0218_F TAATTTATATCTTAATATAGGTCGTAGTAGATGGCCAATCAATGCAATCCCTTTCGCAAC

pDA01_F CATGTGTGCCACATTCGGGATCGTAGCTATTGCGCAGTTATCAAGAACGGCATCATTAAG
 pbpra0218_F CATGTGTGCCACATTCGGGATCGTAGCTATTGCGCAGTTATCAAGAACGGCATCATTAAG

pDA01_F CGTGAAATTACAAGCAGCCTTAGCACTCACGTTGAGCCTCACAGCCATCATACTCACAGA
 pbpra0218_F CGTGAAATTACAAGCAGCCTTAGCACTCACGTTGAGCCTCACAGCCATCATACTCACAGA

pDA01_F AACACGGGGGATTGGCTGGGTTTACCATCACCGCGGCCATCATTCTTTTGCTCAAAGT
 pbpra0218_F AACACGGGGGATTGGCTGGGTTTACCATCACCGCGGCCATCATTCTTTTGCTCAAAGT

pDA01_F CAAAAATAAAGGCATCAACTGGAAGTATGCCACTATTGGGCTTGCCGCGTTTCTATGTAT
 pbpra0218_F CAAAAATAAAGGCATCAACTGGAAGTATGCCACTATTGGGCTTGCCGCGTTTCTATGTAT

pDA01_F CGGTTTCATTGCTAAACCACAACCTAGAACAACGTATTGAACAAACACAAACAGAAATAAA
 pbpra0218_F CGGTTTCATTGCTAAACCACAACCTAGAACAACGTATTGAACAAACACAAACAGAAATAAA

pDA01_F ATCAATTGCATCGGGTAACTTAG-CAC-TCTATCGGTTTACGTTTTCAA-TGTGGAACT
 pbpra0218_F ATCAATTGCATCGGGTAACTTAGCACCCTCTATCGGTTTACGTTTTCAAATGTGGAACT

pDA01_F GGCACCTGATATGCTTAAAGATA--TACATCT--AGTTTAGGGGATA-GCATGCAT-ACG
 pbpra0218_F GGCACCTGATATGCTTAAAGATAATTACATCTTAGGTTTAGGGGATAAGCATGCATTACG

pDA01_F ATTTGACAA--CTTACA-TCAGA--CTTGTTCT--GAAAACTATTCA--CTTCAGCC-GC
 pbpra0218_F ATTTGACAAACTTTACAATCAAGACTTTGTTTCGAAAAAACTATTCAACTTCAGCCCGC

pDA01_F ACACTAC-ATATC--ATATTTAGACA-GCTGAATAAA-GTGGT-TGTTTCG--TTATTATT
 pbpra0218_F ACACTACCATAATCAATATTTAGACAAGCTGATTAAAAAGTGGTATGTTTCGGTTTATTATT

pDA01_F GGTTTAG--ATTACTTATTGTTCCAATAATAACAATCAAAAC--TTAG--ATAGAAGAA
 pbpra0218_F GTTTTAGTATTACTTATTGTTCCAATAATAACAATCAAAACATTAGTAATAGAAAGA

pDA01_F -----CCACTTA-AGGAA--AGCAGCCC--TTGCCAT-GCTGCGT-ACCCAGTTAAC--
 pbpra0218_F AAACACCAACTTATAGGTATTAGCAGCCTCTTTGCTATCGCTGCGTTAACCAGTGTACCA

pDA01_F TTGAACT-TGGTCACCGATTACAATTATATATATGGGTGGT-----CCTGACAAATTC
 pbpra0218_F TTGAATCATGGTCAGACACTGCTTACATATTTATTATTGGTTTGTGGCTTAGCAAATCAC

pDA01_F CTTAAA-CTCCGA-----
 pbpra0218_F CATAAAACCTCAAAGAAGAACAATTGCTAA

Figure 72: Forward sequence alignment of *pbpra0218* and pDA01

Clustalw and Boxshade alignment of *P. profundum* SS9 *pbpra0218* and pDA01. Grey box indicates the P_{BAD} promoter and the Red box indicated the ribosomal binding site (RBS).

pDA01_R CCGGGTAACAGAAATCACTATAAGGGCGATTTGGAGCTCCACCGCGGTGGCGGCCGCTCT
pbpra0218_R -----

pDA01_R AGATTAGCAATTGTTCTTCTTTGAAGTTTTATGGTGATTGCTAAGCCACAAACCAATAA
pbpra0218_R --TTAGCAATTGTTCTTCTTTGAAGTTTTATGGTGATTGCTAAGCCACAAACCAATAA

pDA01_R TAAATATGTAAGCAGTGTCTGACCATGATTCAATGGTACATCGGTTAACGCAGCGATAGC
pbpra0218_R TAAATATGTAAGCAGTGTCTGACCATGATTCAATGGTACATCGGTTAACGCAGCGATAGC

pDA01_R AAAGAGGCTGCTAATACCTATAAGTTGGTGTTTTCTTTCTATTACTAATGTTTTTGATTG
pbpra0218_R AAAGAGGCTGCTAATACCTATAAGTTGGTGTTTTCTTTCTATTACTAATGTTTTTGATTG

pDA01_R TATTATTGGAACAATAAGTAATACTAAAAACAATAATAAACCGAACATACCACTTTTAAT
pbpra0218_R TATTATTGGAACAATAAGTAATACTAAAAACAATAATAAACCGAACATACCACTTTTAAT

pDA01_R CAGCTTGTCTAAATATTGATTATGGTAGTGTGCGGGCTGGAAGTTGAATAGTTTTTTCGA
pbpra0218_R CAGCTTGTCTAAATATTGATTATGGTAGTGTGCGGGCTGGAAGTTGAATAGTTTTTTCGA

pDA01_R AACAAAGTCTTGATTGTAAAGTTTGTCAAATCGTAATGCATGCTTATCCCCTAAACCTAA
pbpra0218_R AACAAAGTCTTGATTGTAAAGTTTGTCAAATCGTAATGCATGCTTATCCCCTAAACCTAA

pDA01_R GATGTAATTATCTTTAAGCATATCAGGTGCCAGTTTCCACATTTGAAAACGTAAACCGAT
pbpra0218_R GATGTAATTATCTTTAAGCATATCAGGTGCCAGTTTCCACATTTGAAAACGTAAACCGAT

pDA01_R AGAGGTGCCTAAGTTACCCGATGCAATTGATTTTAATTTCTGTTTGTGTTTGTTCATAAC
pbpra0218_R AGAGGTGCCTAAGTTACCCGATGCAATTGATTTTA- TTTCTGTTTGTGTTTGTTCATAAC

pDA01_R GTTGTTTCTAGTTTGTGGTTTAGCAAAATGAAACCGAACACATATAAACGCGGCAAGCCCA
pbpra0218_R GTTGTT-CTAGTT-GTGGTTTAGCAA-TGAAACCGA-TACATAGAAACGCGGCAAGCCCA

pDA01_R ATAGTGGGCATACTTCCAGTTGATGCCTTTTATTTTTGACTTTTGAGCAAAAGATGATGGG
pbpra0218_R ATAGTGG-CATACTTCCAGTTGATGCCTTT-ATTTTTGACTTTGAGCAAAAGAATGATGG

pDA01_R CCGCAGTGATGGTAAAACCCAAACC AAAATCCCCCGTGTTTCTGTGAGTATGATTGCCTGT
pbpra0218_R CCGCGGTGATGGTAAAACCCAGCC- AAATCCCCCGTGTTTCTGTGAGTATGAT-GGCTGT

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pDA01_R      GTTGTTTCTAGTTTGTGGTTTAGCAAATGAAACCGAACACATAATAACGCGGCAAGCCCA
pbpra0218_R  GTTGTT-CTAGTT-GTGGTTTAGCAA-TGAAACCGA-TACATAGAAACGCGGCAAGCCCA

pDA01_R      ATAGTGGGCATACTTCCAGTTGATGCCTTTATTTTGGACTTTTGAGCAAAGATGATGGG
pbpra0218_R  ATAGTGG-CATACTTCCAGTTGATGCCTTT-ATTTTGGACTTTGAGCAAAGAAATGATGG

pDA01_R      CCGCAGTGATGGTAAAACCCAAACC AAAATCCCCCGTGTTTCTGTGAGTATGATTGCCTGT
pbpra0218_R  CCGCGGTGATGGTAAAACCCAGCC-AAAATCCCCCGTGTTTCTGTGAGTATGAT-GGCTGT

pDA01_R      GAGGCTCA-CGTGAGTGCTAAGGCTGCTTGTAATTTACGCTTAATGATGCCGTTCT-GA
pbpra0218_R  GAGGCTCAACGTGAGTGCTAAGGCTGCTTGTAATTTACGCTTAATGATGCCGTTCTTGA

pDA01_R      TACTGTGCAATAGCTACGATCCCGAATGTGGC-CACATGGTTGCCAAACTGATTGCATT
pbpra0218_R  TAACTGCGCAATAGCTACGATCCCGAATGTGGCAACACATGGTTGCGAAAGGATTGCATT

pDA01_R      GAT-GGCCATCTACTACGATCTATAT-AAGATCTTAACATAGTAACACGCTC-----
pbpra0218_R  GATTGGCCATCTACTACGACCTATATTAAAGATATAAATTATAGTAACACGCATATGCTAA

pDA01_R      -----
pbpra0218_R  GCAGAGAGATGATGCTAAGAGTGTTAACCCTAATAAGATTCTTTGAGTTAGCAGCTCTCT

pDA01_R      -----
pbpra0218_R  CGGAAAACAAATTAAAAATAAGGATGCACCAATCAAGGCACGAATTTCTCGTGAGCTACC

```

Figure 73: Reverse sequence alignment of *pbpra0218* and pDA01

Clustalw and Boxshade alignment of *P. profundum* SS9 *pbpra0218* and pDA01. Blue box indicates the Stop site.

Cloning only *pbpra0218* did not complement the cold temperature sensitivity or fully complement the LPS phenotype. Therefore the adjoining gene, *pbpra0217*, was also cloned due to the fact that these genes overlapped by 6 bp (Figure 24). The PCR product including both genes was expected to be 1934 bp.

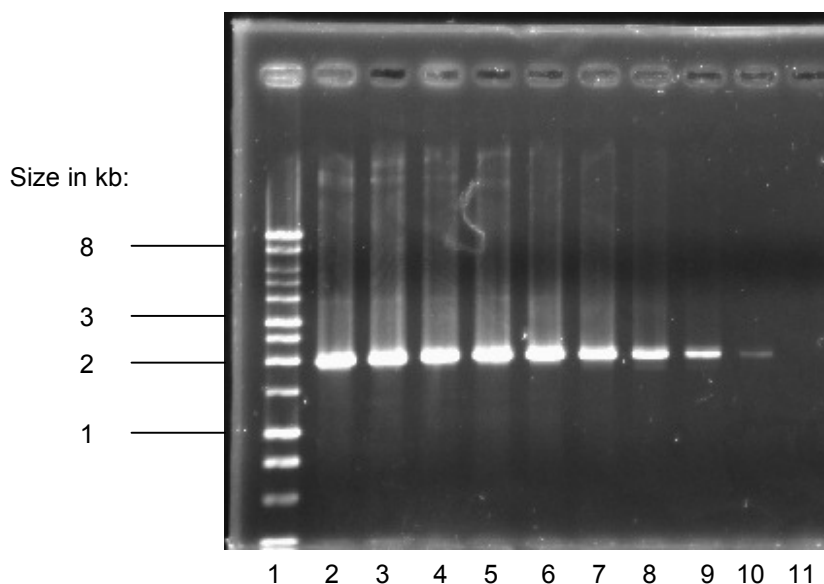


Figure 74: Temperature gradient PCR of *pbpra0218-0217*

The genes *pbpra0218-0217* were amplified using the primers Pbpra0218-F_ *EcoRI* and Pbpra02180-217R_ *XbaI* by PCR using a temperature gradient to find the optimum temperature, separated on 0.8% w/v agarose gel. 1 kb DNA ladder (Promega) (1), and the following annealing temperatures: 58.1°C (2), 58.5°C (3), 59.1°C (4), 59.9°C (5), 60.8°C (6), 61.7°C (7), 62.6°C (8), 63.4°C (9), 64.1°C (10), -ve control no template DNA (11). 2 minutes 30 seconds extension time.

The PCR product was digested with *EcoRI* and *XbaI* and ligated into double digested pFL190 before being transformed into *E. coli* DH5 α . Transformants were purified twice on LB agar with 100 $\mu\text{g.ml}^{-1}$ streptomycin and the resulting colonies were screened by restriction digest (Figure 75). The agarose gel showed that 3 out of 7 randomly chosen clones contained an insert of approximately the correct size (Figure 75 lanes 3, 11 and 13).

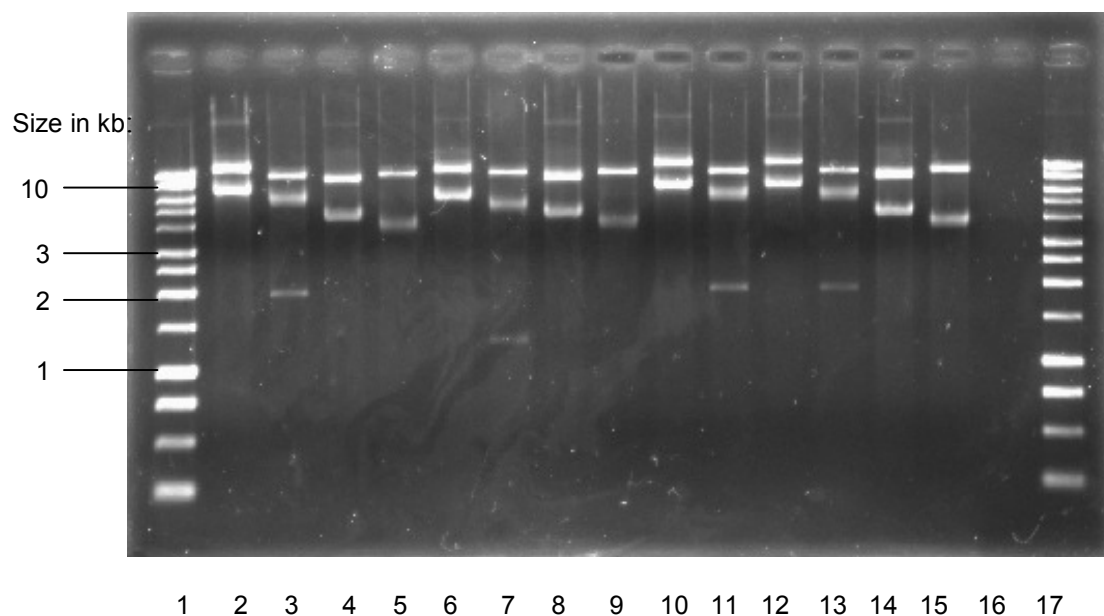


Figure 75: Screening *E. coli* DH5 α pFL190+*pbpra0218-0217* clones by restriction digest

Following ligation and transformation, those clones growing on LB with streptomycin were purified and the plasmids extracted. The plasmids were then digested with *Xba*I and *Eco*RI and separated on 0.8% w/v agarose gel. 1Kb DNA ladder (NEB) (lanes 1 and 17), clones 1-7 (lanes 2, 4, 6, 8, 10, 12 and 14) double digested (lanes 3, 5, 7, 9, 11, 13 and 15).

Unfortunately, sequencing of the *pbpra0218-0217* clones showed that the sequences were significantly error prone (data not shown). A number of clones were sequenced with the same result each time. Unfortunately the PCR and cloning of *pbpra0218-0217* was not repeated at this time.

Appendix B – Cloning of *S. Typhimurium* O-antigen ligase

The *Salmonella* SARC 1 O-antigen ligase gene was digested from pWQ310 using *Eco*RI and *Xba*I to release a fragment approximately 1.2 kb and empty plasmid approximately 5 kb (Figure 76). The fragment was purified from the gel and ligated in a 4:1 ratio with pFL190. The ligation of *Salmonella* SARC 1 O-antigen ligase gene with pFL190 and subsequent transformation into *Salmonella* resulted in only 2 colonies. After these colonies were screened by restriction digest they were both found to contain pFL190 with a fragment of approximately the correct size indicating *Salmonella* SARC 1 O-antigen ligase gene (Figure 77). The construct was then named pDA03 and transformed into *S. Typhimurium* LT2 and SL3749.

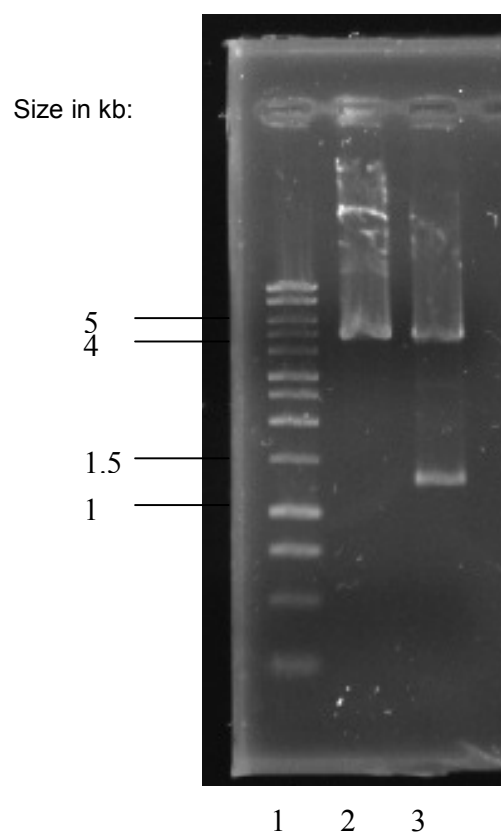


Figure 76: Restriction digest of pWQ310 containing of O-antigen ligase from *Salmonella*
The plasmid was digested with *Xba*I and *Eco*RI and separated on 0.8 % (w/v) agarose gel. 1 Kb DNA ladder (Promega) (lane 1), pWQ310 uncut (2), pWQ310 double digested (3).

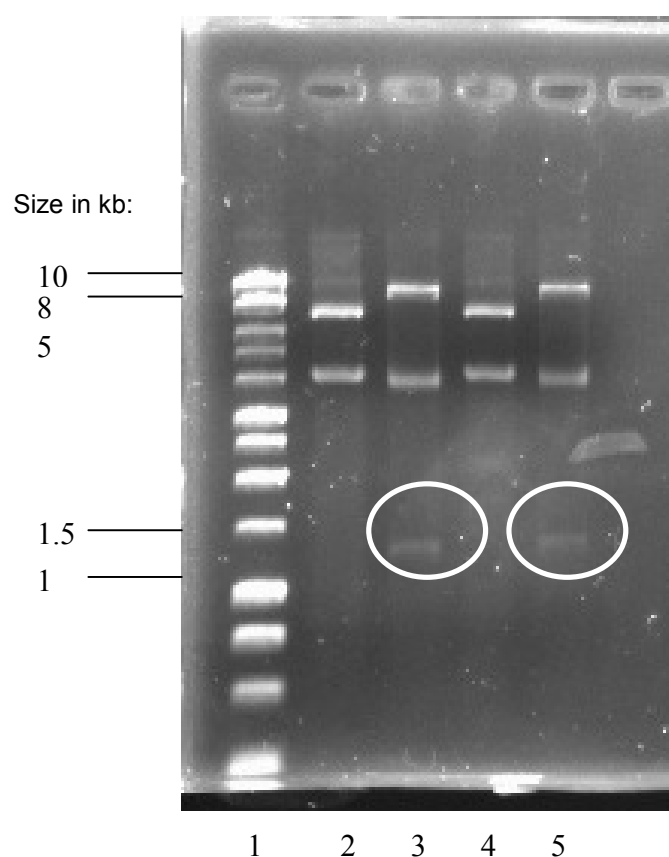


Figure 77: Restriction digest of pFL190 containing of O-antigen ligase from *Salmonella*, pDA03

The plasmid was digested with *Xba*I and *Eco*RI and separated on 0.8 % (w/v) agarose gel. 1 Kb DNA ladder (Promega) (lane 1), clone 1 uncut (2), clone 1 double digested (3), clone 2 uncut (3), clone 2 double digested (4). White circles highlight the 1.3 kb O-antigen ligase gene.

Appendix C – Cloning of *P. profundum* SS9 tyrosine protein kinase, *pbpra2686*

Firstly, *pbpra2686* was amplified by PCR resulting in a fragment of the correct size (2158 bp). To find the optimum annealing temperature a gradient was used, this resulted in secondary bands (Figure 78), therefore the PCR product was excised from the gel using QiaQuick Gel Extraction kit and used for subsequent restriction digests.

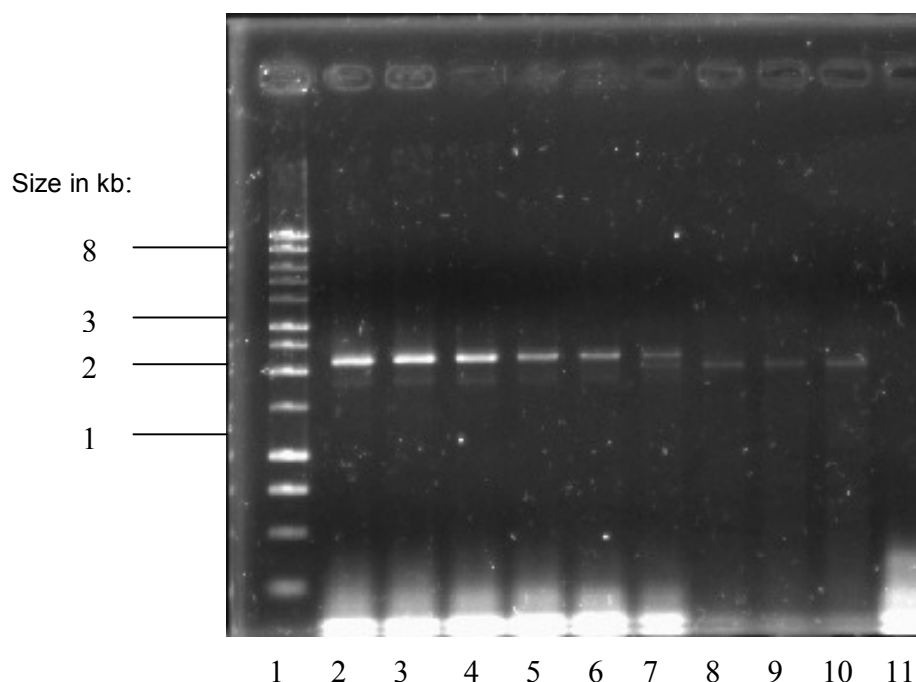


Figure 78: Temperature gradient PCR of *pbpra2686*

The genes *pbpra2686* were amplified using the primers Pbpra2686-16F_ *Bsa*I and Pbpra2686-R_ *Xba*I by PCR using a temperature gradient and an additional 2.5% v/v DMSO to find the optimum temperature, separated on 0.8% w/v agarose gel. 1 kb DNA ladder (Promega) (1), and the following annealing temperatures: 58.1°C (2), 58.5°C (3), 59.1°C (4), 59.9°C (5), 60.8°C (6), 61.7°C (7), 62.6°C (8), 63.4°C (9), 64.1°C (10), ~ve control no template DNA (11). 2 minutes 30 seconds extension time.

The *pbpra2686* PCR product was double digested with *Xba*I and *Eco*RI and ligated overnight at 4°C in a 4:1 ratio with double digested pFL190. The ligation mixture was then added to 25 µl competent cells from NEB and a basic transformation

procedure was followed. Successful transformants were purified twice on LB agar with $100 \mu\text{g.ml}^{-1}$ streptomycin before the plasmids were purified and screened by restriction digest. One such restriction digest screened 7 clones and compared the uncut to the cut plasmids (Figure 79). Clones containing an insert of the correct size can be seen in lanes 9, 13 and 15.

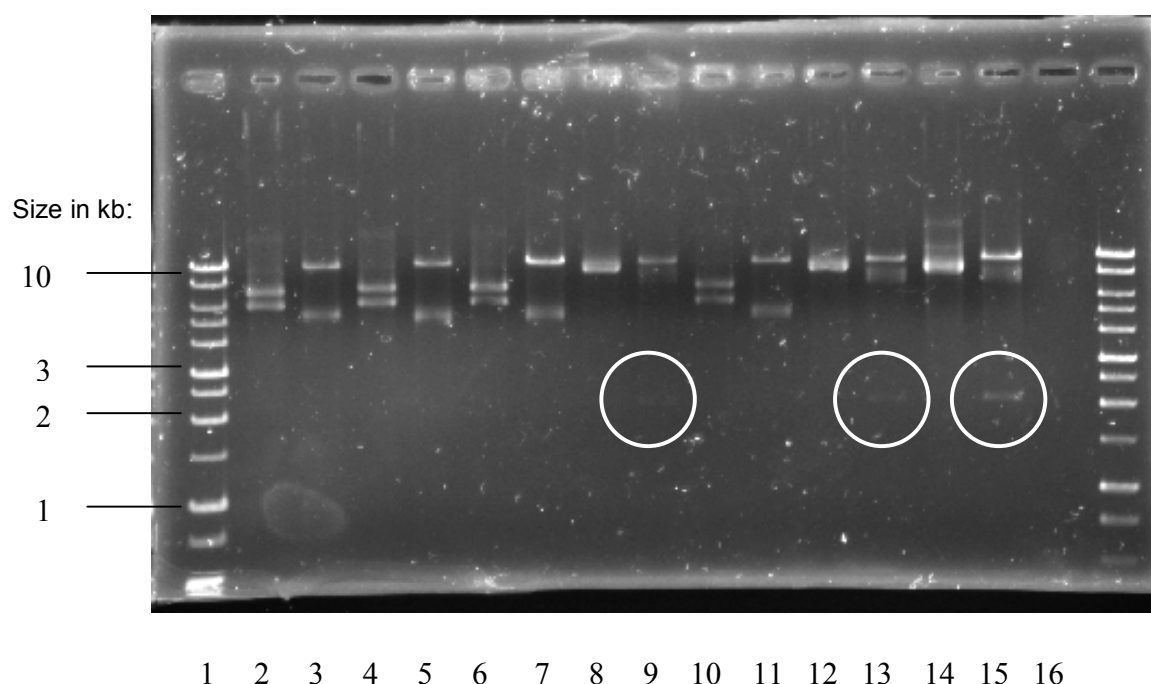


Figure 79: Screening *E. coli* DH5 α pFL190+*pbpra2686* clones by restriction digest

Following ligation and transformation, those clones growing on LB with streptomycin were purified and the plasmids extracted. The plasmids were then digested with *Xba*I and *Eco*RI and separated on 0.8% w/v agarose gel. 1 Kb DNA ladder (NEB) (lanes 1 and 17), clones 1-7 (lanes 2, 4, 6, 8, 10, 12 and 14) double digested (lanes 3, 5, 7, 9, 11, 13 and 15). White circles indicate the fragment band.

Clones containing an insert of approximately the correct size were sequenced forward and backwards using pFL190 sequencing primers (Appendix Figure 80 and Figure 83, respectively) and 2 internal primers 714F and 1134F (Appendix Figure 81

and Figure 82, respectively), this was due to the size of the size of the gene (~2.2 kb) and the length of useful PCR sequences obtained previously. By overlapping the sequences obtained we found that the sequence was accurate and the plasmid was then renamed pDA02. pDA02 was then mated in *P. profundum* SS9R and FL26 using the mating protocol outlined in methods 2.10.11.

pDA02_F CCGGTCAGTGTCTATAATCACGGCAGAAAGTCCACATTGATTATTTGCACGGCGTCACAC
pbpra2686_F -----

pDA02_F TTTGCTATGCCATAGCATTTTTATCCATAAGATTAGCGGATCCTACCTGACGCTTTTTAT
pbpra2686_F -----

pDA02_F CGCAACTCTCTACTGTTTCTCCATAACCCGTTTTTTTGGGCTAGCGAATTCAGTTGAAAA
pbpra2686_F -----TAGTTGAAAA

pDA02_F ACATTTATGAGCAACATAAAAAACACAAGCGTAATGCAAAATAAAACGGATGAAATAGAT
pbpra2686_F ACATTTATGAGCAACATAAAAAACACAAGCGTAATGCAAAATAAAACGGATGAAATAGAT

pDA02_F TTAACCAAACTTTTTGGAACACTAATAGATAGTCGATGGACTATAATTATTATCACTTTT
pbpra2686_F TTAACCAAACTTTTTGGAACACTAATAGATAGTCGATGGACTATAATTATTATCACTTTT

pDA02_F ATATTTGCTATAATTGGTGTGCGATATGCAGTTTTTTCAACGCCAATATATAAAGCCGAC
pbpra2686_F ATATTTGCTATAATTGGTGTGCGATATGCAGTTTTTTCAACGCCAATATATAAAGCCGAC

pDA02_F TCACTAATTCAAGTTGAGAAAAAAGTAGTGGTGTTCAGTACTTGGTGAAATGGGAGAG
pbpra2686_F TCACTAATTCAAGTTGAGAAAAAAGTAGTGGTGTTCAGTACTTGGTGAAATGGGAGAG

pDA02_F CTTTTTAGTAGTGAATCATCTTCCTCAACTGAAATGGAACATAATTAAATCAAGAATGATA
pbpra2686_F CTTTTTAGTAGTGAATCATCTTCCTCAACTGAAATGGAACATAATTAAATCAAGAATGATA

pDA02_F CTTGAAAAGACTGTTGATAAGATGAACTTAACAACAGTATCTGAACCTGTACTTACTCCA
pbpra2686_F CTTGAAAAGACTGTTGATAAGATGAACTTAACAACAGTATCTGAACCTGTACTTACTCCA

pDA02_F GTTATAGGTAAAGGATTACAAAGATTAAGTGGCGACGAATTGAATATTGATATAGTTCGA
pbpra2686_F GTTATAGGTAAAGGATTACAAAGATTAAGTGGCGACGAATTGAATATTGATATAGTTCGA

pDA02_F TTTGAAATTAATAATAGCCATCTAACTAATTATATATTAAAGGTTTTAGATTCTGATAAA
pbpra2686_F TTTGAAATTAATAATAGCCATCTAACTAATTATATATTAAAGGTTTTAGATTCTGATAAA

pDA02_F GGTTTATATGTAATTTTCGACGAAAATGAGAAAGAAATATTACAAGGTATTACAGGGAGA
pbpra2686_F GGTTTATATGTAATTTTCGACGAAAATGAGAAAGAAATATTACAAGGTATTACAGGGAGA

pDA02_F GCTGTATCAAAAAATGGTATTAATATTTTTATTTCGAAGTATTAATGCTGAAAGTGGGGCA
pbpra2686_F GCTGTATCAAAAAATGGTATTAATATTTTTATTTCGAAGTATTAATGCTGAAAGTGGGGCA

pDA02_F TCATTTAGAGTTTCGAAACGAAGTAAATTAGAAGCAATTCAATGGTTGAATAGTAAGCTA
pbpra2686_F TCATTTAGAGTTTCGAAACGAAGTAAATTAGAAGCAATTCAATGGTTGAATAGTAAGCTA

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pDA02_F      AGTATAGAAGAGTTAGGTAGAACTTCAGGTATTATGCAGCTTACATTTATTGGTAGTAAT
pbpra2686_F  AGTATAGAAGAGTTAGGTAGAACTTCAGGTATTATGCAGCTTACATTTATTGGTAGTAAT

pDA02_F      AAAAAATCAAAATGAAAGCATTTTTAAATAGCATTAAATCATGATTATCTATTACAAAACGTA
pbpra2686_F  AAAAAATCAAAATGAAAGCATTTTTAAATAGCATTAAATCATGATTATCTATTACAAAACGTA

pDA02_F      GAGAGAAGTTCGGCTGAAGCTGAAA-TAGTTTGGTTTTTTT--AAAGAGCATTTACCAAGC
pbpra2686_F  GAGAGAAGTTCGGCTGAAGCTGAAAATAGTTTGGTTTTTTTAAAGAGCATTTACCAAGC

pDA02_F      ATTAATAA-GATTTGGTAATCTCAGAAA-TAG--TAAATATACAGCAG-----AAATGAT
pbpra2686_F  ATTAATAAAGATTTGGTAATCTCAGAAAATAAGTTAAATAATTACAGCAAGAAAATGAT

pDA02_F      TCTATTGATCTAAACT--AGAGCTAAAGCTACTCTTGATGCATT-GTGA--TTAGAAA-G
pbpra2686_F  TCTATTGATCTAAACTTAGAAGCTAAAGCTACTCTTGATGCATTGTGAATTTAGAAAAG

pDA02_F      CA--TCAATCA-CTGACATTTGAGGA--GTGA-ATTGCTCAAAA-TA-ACAAAGACTCAG
pbpra2686_F  CAATTAAATCAACTGACATTTAAAGAAAAGTGAGATTGCTCAAAAATATACAAAAGAACAT

pDA02_F      TTAATA-ATTCTCCTTCTTTGAATAAACGAAA-GTCTTAATTGTATAAGCA-----
pbpra2686_F  CCAGTTTATATATCTCTTCTTGATAAACGAAAAGTCTAAATG-ATAAGCAAGTAGAACT

pDA02_F      -----
pbpra2686_F  AGAAAAACAAATAAAAAAATTACCAATAACTCAAAGAGAAGTTTTGCGGTAAACAAGAGA

pDA02_F      -----
pbpra2686_F  TGTAATGTAAATCAACAGATGTATGTGCAACTTTTAAATAAAGTTCAAGAATTTAACAT

pDA02_F      -----
pbpra2686_F  AGTTAAAGCTAGTACTGTTGGTAATGTTGCAATAATTGATTCTGCAAAATCAACTGCTAG

pDA02_F      -----
pbpra2686_F  AGCAGTAAACCAGTTAAATCTTTGATAGTTATAATATCAACATTGTTAGGCTTGATTAT

```

Figure 80: Forward sequence alignment of *pbpra2686* and pDA02

Clustalw and Boxshade alignment of *P. profundum* SS9 *pbpra2686* and pDA02. Grey box indicates the P_{BAD} promoter and Red box indicated the ribosomal binding site (RBS).

pDA02_IntF -----CTT**TTAATTGGTAGTAATAAAA-T**
pbpra2686_F GAAGAGTTAGGTAGAACTTCAGGTATTATGCAGCTTACAT**TTTATTGGTAGTAATAAAAAT**

pDA02_IntF **CAAAATGAAAGCATTTTAAATAGCATTAAATCATGATTATCTATTACAAAACGTAGAGAGA**
pbpra2686_F **CAAAATGAAAGCATTTTAAATAGCATTAAATCATGATTATCTATTACAAAACGTAGAGAGA**

pDA02_IntF **AGTTCGGCTGAAGCTGAAAATAGTTTGGTTTTTTTAAAAGAGCATTACCAAGCATTAAA**
pbpra2686_F **AGTTCGGCTGAAGCTGAAAATAGTTTGGTTTTTTTAAAAGAGCATTACCAAGCATTAAA**

pDA02_IntF **AAAGATTTGGTAATCTCAGAAAAATAAGTTAAATAATTACCAGCAAGAAAATGATTCTATT**
pbpra2686_F **AAAGATTTGGTAATCTCAGAAAAATAAGTTAAATAATTACCAGCAAGAAAATGATTCTATT**

pDA02_IntF **GATCTAACTTAGAAGCTAAAGCTACTCTTGATGCATTTGTGAATTTAGAAAAGCAATTA**
pbpra2686_F **GATCTAACTTAGAAGCTAAAGCTACTCTTGATGCATTTGTGAATTTAGAAAAGCAATTA**

pDA02_IntF **AATCAACTGACATTTAAAGAAAGTGAGATTGCTCAAAAATATACAAAAGAACATCCAGTT**
pbpra2686_F **AATCAACTGACATTTAAAGAAAGTGAGATTGCTCAAAAATATACAAAAGAACATCCAGTT**

pDA02_IntF **TATATATCTCTTCTTGATAAACGAAAAGTCCTAAATGATAAGCAAGTAGAACTAGAAAAA**
pbpra2686_F **TATATATCTCTTCTTGATAAACGAAAAGTCCTAAATGATAAGCAAGTAGAACTAGAAAAA**

pDA02_IntF **CAAAATAAAAAAATTACCAATAACTCAAAGAGAAGTTTTGCGGTTAACAAGAGATGTAAAT**
pbpra2686_F **CAAAATAAAAAAATTACCAATAACTCAAAGAGAAGTTTTGCGGTTAACAAGAGATGTAAAT**

pDA02_IntF **GTAAATCAACAGATGTATGTGCAACTTTTAAATAAAGTTCAAGAATTTAACATAGTTAAA**
pbpra2686_F **GTAAATCAACAGATGTATGTGCAACTTTTAAATAAAGTTCAAGAATTTAACATAGTTAAA**

pDA02_IntF **GCTAGTACTGTTGGTAATGTTTCAATAATTGATTCTGCAAAATCAACTGCTAGAGCAGTA**
pbpra2686_F **GCTAGTACTGTTGGTAATGTTTCAATAATTGATTCTGCAAAATCAACTGCTAGAGCAGTA**

pDA02_IntF **AAACCAGTTAAATCTTTGATAGTTATAATATCAACATTGTTAGGCTTGATTATTGGGGTT**
pbpra2686_F **AAACCAGTTAAATCTTTGATAGTTATAATATCAACATTGTTAGGCTTGATTATTGGGGTT**

pDA02_IntF **TTCTATACATTATTTAAATCATCCTTGAATAAGGGGGTAGAAAAATCCTGATGAAATTGAA**
pbpra2686_F **TTCTATACATTATTTAAATCATCCTTGAATAAGGGGGTAGAAAAATCCTGATGAAATTGAA**

pDA02_IntF **AAGCTAGGAATACCTGTATATGCAAGTATTCCTATGTCCGATTGGCAAATAAAGATAGAT**
pbpra2686_F **AAGCTAGGAATACCTGTATATGCAAGTATTCCTATGTCCGATTGGCAAATAAAGATAGAT**

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pDA02_IntF AAAGAACAATCTAAATCAAAAAATTTAAAAATAGATAAAACATTACTTGCAGTTGTAAAC
pbpra2686_F AAAGAACAATCTAAATCAAAAAATTTAAAAATAGATAAAACATTACTTGCAGTTGTAAAC

pDA02_IntF CCAGCTGATTTATCGATTGAAGCGATAAGAAGCCTGCGTACAAGTTTACATTTTGCAATG
pbpra2686_F CCAGCTGATTTATCGATTGAAGCGATAAGAAGCCTGCGTACAAGTTTACATTTTGCAATG

pDA02_IntF ATGGAAGCTAAAAATAATATTTTAAATGATATCGGGACCAAGCCCAAGTATCGGGAAGTCT
pbpra2686_F ATGGAAGCTAAAAATAATATTTTAAATGATATCGGGACCAAGCCCAAGTATCGGGAAGTCT

pDA02_IntF TTTGTATCAGCTAATATGGCTTCTGTTATAGCGAAATCAG-ATCTAAAATA-TCGT-ATC
pbpra2686_F TTTGTATCAGCTAATATGGCTTCTGTTATAGCGAAATCAGGATCTAAAATAATCGTTATC

pDA02_IntF GATGCTGAATATGAGAAAGG-ACGCATG--ACGTCAGATGG-CGTA-CTAGCAA--CTG-
pbpra2686_F GATGCTGA-TATGAGAAAGGACGCATGGAACGTCAGATGGCGTAACCTAGCAAACCTGG

pDA02_IntF -CTATCCGGT-ATT--AGTGATCAGT--AATATCGATAATATAGTAAAGC--ACTG----
pbpra2686_F TTTATCCGGTTATTAAAGTGATCAAGTAAATATCGATAATATAGTAAAGCAACTGGCGT

pDA02_IntF CGTGAATTCAA--TTTATGCGAGAGGT--ACTCA--CTAAC--ATCTGGAT----AT
pbpra2686_F TGAAAATTTAGATTTTATTGCGAGAGGTGATACTCCACCTAACCCATCTGAGTTATTGAT

pDA02_IntF GAGCCCAAG---TAGG--TCTATTGGATGG---CTCGAAATAGAATGTCTCTGACCCAAT
pbpra2686_F GCACCCAAGGTTTAAGGTTCTATTGGATTGGGCTTCGGAAAATTATGATATTGTTCTTTAT

pDA02_IntF CCGGTCAATTCCAATGATCAGGA-----
pbpra2686_F TGATACCCCTCCAATCTTGCTGTTACAGATTACAGCAATAGTTGGATCTCATGCGGGAAC

pDA02_IntF -----
pbpra2686_F TACCTTGCTTGTTGGTCGCTTTGGTCAGAATACGGCAAAAGAGATAGAAGTAGCCAAGCA

pDA02_IntF -----
pbpra2686_F AAGATTTGAACAAAAATGGAATTCAAGTGAAAGGCTTTATTTTGAATGCGGTTATAAAGAA

pDA02_IntF -----
pbpra2686_F AGCAGGTAATAGTTATGGATATTATAATTATAGCTATGAATAA

```

Figure 81: Forward sequence alignment of *pbpra2686* internal fragment 714 and pDA02
 Clustalw and Boxshade alignment of *P. profundum* SS9 *pbpra2686* and pDA02.

pDA02_Int_F -----
pbpra2686_F CAAATAAAAAAATTACCAATAACTCAAAGAGAAAGTTTTGCGGTTAACAAGAGATGTAAAT

pDA02_Int_F - - - - GAAGGGAATGTATGTGCACTTTT - - AATAAAGTTCAAGAATTTAACATAGTTAAA
pbpra2686_F GTAAATCAACAGATGTATGTGCAACTTTTAAATAAAGTTCAAGAATTTAACATAGTTAAA

pDA02_Int_F GCTAGTACTGTTGGTAATGTTTCAATAAATTGATTCTGCAAAATCAACTGCTAGAGCAGTA
pbpra2686_F GCTAGTACTGTTGGTAATGTTTCAATAAATTGATTCTGCAAAATCAACTGCTAGAGCAGTA

pDA02_Int_F AAACCAGTTAAATCTTTGATAGTTATAATATCAACATTGTTAGGCTTGATTATTGGGGTT
pbpra2686_F AAACCAGTTAAATCTTTGATAGTTATAATATCAACATTGTTAGGCTTGATTATTGGGGTT

pDA02_Int_F TTCTATACATTATTTAAATCATCCTTGAATAAGGGGGTAGAAAATCCTGATGAAATTGAA
pbpra2686_F TTCTATACATTATTTAAATCATCCTTGAATAAGGGGGTAGAAAATCCTGATGAAATTGAA

pDA02_Int_F AAGCTAGGAATACCTGTATATGCAAGTATTCCTATGTCCGATTGGCAAATAAAGATAGAT
pbpra2686_F AAGCTAGGAATACCTGTATATGCAAGTATTCCTATGTCCGATTGGCAAATAAAGATAGAT

pDA02_Int_F AAAGAACAATCTAAATCAAAAAATTTAAAAATAGATAAAACATTACTTGCAGTTGTAAAC
pbpra2686_F AAAGAACAATCTAAATCAAAAAATTTAAAAATAGATAAAACATTACTTGCAGTTGTAAAC

pDA02_Int_F CCAGCTGATTTATCGATTGAAGCGATAAGAAGCCTGCGTACAAGTTTACATTTTGCAATG
pbpra2686_F CCAGCTGATTTATCGATTGAAGCGATAAGAAGCCTGCGTACAAGTTTACATTTTGCAATG

pDA02_Int_F ATGGAAGCTAAAAATAATATTTTAATGATATCGGGACCAAGCCCAAGTATCGGGAAGTCT
pbpra2686_F ATGGAAGCTAAAAATAATATTTTAATGATATCGGGACCAAGCCCAAGTATCGGGAAGTCT

pDA02_Int_F TTTGTATCAGCTAATATGGCTTCTGTTATAGCGAAATCAGGATCTAAAATAATCGTTATC
pbpra2686_F TTTGTATCAGCTAATATGGCTTCTGTTATAGCGAAATCAGGATCTAAAATAATCGTTATC

pDA02_Int_F GATGCTGATATGAGAAAGGGACGCATGGAACGTCAGATGGGCGTAACTAGCAAACCTGGT
pbpra2686_F GATGCTGATATGAGAAAGGGACGCATGGAACGTCAGATGGGCGTAACTAGCAAACCTGGT

pDA02_Int_F TTATCCGGTTATTTAAGTGATCAAGTAAATATCGATAATATAGTAAAAGCAACTGGCGTT
pbpra2686_F TTATCCGGTTATTTAAGTGATCAAGTAAATATCGATAATATAGTAAAAGCAACTGGCGTT

pDA02_Int_F GAAAATTTAGATTTTATTGCGAGAGGTGATACTCCACCTAACCCATCTGAGTTATTGATG
pbpra2686_F GAAAATTTAGATTTTATTGCGAGAGGTGATACTCCACCTAACCCATCTGAGTTATTGATG


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pDA02_Int_F CACCCAAGGTTTAAGGTTCTATTGGATTGGGCTTCGGAAAATTATGATATTGTTCTTATT
pbpra2686_F CACCCAAGGTTTAAGGTTCTATTGGATTGGGCTTCGGAAAATTATGATATTGTTCTTATT

pDA02_Int_F GATACCCCTCCAATTCTTGCTGTTACAGATTCAGCAATAGTTGGATCTCATGCGGGAAC
pbpra2686_F GATACCCCTCCAATTCTTGCTGTTACAGATTCAGCAATAGTTGGATCTCATGCGGGAAC

pDA02_Int_F ACCTTGCTTGTTGGTCGCTTTGGTCAGAATACGGCAAAAAGAGATAGAAGTAGCCAAGCAA
pbpra2686_F ACCTTGCTTGTTGGTCGCTTTGGTCAGAATACGGCAAAAAGAGATAGAAGTAGCCAAGCAA

pDA02_Int_F AGATTTGAACAAAATGGAATTCAAGTGAAAGGCTTTATTTTGAATGCGGTTATAAAGAAA
pbpra2686_F AGATTTGAACAAAATGGAATTCAAGTGAAAGGCTTTATTTTGAATGCGGTTATAAAGAAA

pDA02_Int_F GCAG- TAATAGTTATGGATATTATAATTATAGCTATGAATAA TCTAGAGCGGCCGCCACC
pbpra2686_F GCAGGTAATAGTTATGGATATTATAATTATAGCTATGAATAA- - - - -

pDA02_Int_F GCGGTGGAGCTCCAATTCGCCCTATAGTGAGTCGTATTACGCGCGCTCACTGGCCGTCGT
pbpra2686_F - - - - -

pDA02_Int_F TTACACGTCGTGACTGGGAAAACCCTGACGTACCAACTATCGCTTGAGCCATCCCCCTT
pbpra2686_F - - - - -

pDA02_Int_F TCGCAGCAGATCTATGCAGAGCAGAGAGCATACATCTGGAGCAAGCAGAAGCGGCCCTAT
pbpra2686_F - - - - -

pDA02_Int_F GGGAGCTTGTCGC
pbpra2686_F - - - - -

```

Figure 82: Forward sequence alignment of *pbpra2686* internal fragment 1134 and pDA02
 Clustalw and Boxshade alignment of *P. profundum* SS9 *pbpra2686* and pDA02. Blue box indicates the Stop site.

pDA02_R TGGGGGAACGAATAAATATAGGGCGATTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGATT
pbpra2686_R -----

pDA02_R TTCATAGCTATAATTATAATATCCATAACTATTACCTGCTTTCTTTATAACCGCATTC
pbpra2686_R TTCATAGCTATAATTATAATATCCATAACTATTACCTGCTTTCTTTATAACCGCATTC

pDA02_R AAAATAAAGCCTTTCACCTGAATTCCATTTTGTTCAAATCTTTGCTTGGCTACTTCTATC
pbpra2686_R AAAATAAAGCCTTTCACCTGAATTCCATTTTGTTCAAATCTTTGCTTGGCTACTTCTATC

pDA02_R TCTTTTGCCGTATTCTGACCAAAGCGACCAACAAGCAAGGTAGTTCCCGCATGAGATCCA
pbpra2686_R TCTTTTGCCGTATTCTGACCAAAGCGACCAACAAGCAAGGTAGTTCCCGCATGAGATCCA

pDA02_R ACTATTGCTGAATCTGTAACAGCAAGAATTGGAGGGGTATCAATAAGAACAATATCATAA
pbpra2686_R ACTATTGCTGAATCTGTAACAGCAAGAATTGGAGGGGTATCAATAAGAACAATATCATAA

pDA02_R TTTTCCGAAGCCCAATCCAATAGAACCTTAAACCTTGGGTGCATCAATAACTCAGATGGG
pbpra2686_R TTTTCCGAAGCCCAATCCAATAGAACCTTAAACCTTGGGTGCATCAATAACTCAGATGGG

pDA02_R TTAGGTGGAGTATCACCTCTCGCAATAAAATCTAAATTTTCAACGCCAGTTGCTTTTACT
pbpra2686_R TTAGGTGGAGTATCACCTCTCGCAATAAAATCTAAATTTTCAACGCCAGTTGCTTTTACT

pDA02_R ATATTATCGATATTTACTTGATCACTTAAATAACCGGATAAACCAGGTTTGCTAGTTACG
pbpra2686_R ATATTATCGATATTTACTTGATCACTTAAATAACCGGATAAACCAGGTTTGCTAGTTACG

pDA02_R CCCATCTGACGTTCCATGCGTCCCTTTCTCATATCAGCATCGATAACGATTATTTTAGAT
pbpra2686_R CCCATCTGACGTTCCATGCGTCCCTTTCTCATATCAGCATCGATAACGATTATTTTAGAT

pDA02_R CCTGATTTTCGCTATAACAGAAGCCATATTAGCTGATACAAAAGACTTCCCGATACTTGGG
pbpra2686_R CCTGATTTTCGCTATAACAGAAGCCATATTAGCTGATACAAAAGACTTCCCGATACTTGGG

pDA02_R CTTGGTCCCGATATCATTAATAATATTATTTTAGCTTCCATCATTGCAAAATGTAACTT
pbpra2686_R CTTGGTCCCGATATCATTAATAATATTATTTTAGCTTCCATCATTGCAAAATGTAACTT

pDA02_R GTACGCAGGCTTCTTATCGCTTCAATCGATAAATCAGCTGGGTTTACAACGCAAGTAAT
pbpra2686_R GTACGCAGGCTTCTTATCGCTTCAATCGATAAATCAGCTGGGTTTACAACGCAAGTAAT

pDA02_R GTTTTATCTATTTTAAATTTTGTGATTTAGATTGTTCTTTATCTATCTTTATTTGCCAA
pbpra2686_R GTTTTATCTATTTTAAATTTTGTGATTTAGATTGTTCTTTATCTATCTTTATTTGCCAA

pDA02_R TCGGACATAGGAATACTTGATATACAGGTATTCCTAGCTTTTCA - TTTTCATCAGGATTT
pbpra2686_R TCGGACATAGGAATACTTGATATACAGGTATTCCTAGCTTTTCA TTTTCATCAGGATTT

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pDA02_R      TCTACCCCCTTATTCAAGGATGATTTTAATAATGTATAGAAAACCCCAATAATCAAGCC
pbpra2686_R  TCTACCCCCTTATTCAAGGATGATTTTAATAATGTATAGAAAACCCCAATAATCAAGCC

pDA02_R      TAACAATGTCGATATTATAACTATCAAAGATTTAACTGGGTTTACTGCTCTAGCAGTAGA
pbpra2686_R  TAACAATGTTGATATTATAACTATCAAAGATTTAACTGGGTTTACTGCTCTAGCAGTTGA

pDA02_R      TTTTGCAGAATCAATTATTTCGAACATTACCAACAGTACTAGCTTTAACCATGTGTAAATTC
pbpra2686_R  TTTTGCAGAATCAATTATTTCGAACATTACCAACAGTACTAGCTTTAACCATGTGTAAATTC

pDA02_R      T- GAACTTTATT- AAAAGTTGCACATCC- TCTGTTTGATTTTATT- AC- TCTCTTGTTAA
pbpra2686_R  TTGAACTTTATTTAAAAGTTGCACATACATCTGTTGATTTACATTTACATCTCTTGTTAA

pDA02_R      CCACGAAC-----
pbpra2686_R  CCGCAAAACTTCTCTTTGAGTTATTGGTAATTTTTTTATTTGTTTTCTAGTTCTACTTG

pDA02_R      -----
pbpra2686_R  CTTATCATTTAGGACTTTTCGTTTATCAAGAAGAGATATATAAACTGGATGTTCTTTTGT

pDA02_R      -----
pbpra2686_R  ATATTTTTTGAGCAATCTCACTTTCTTTAAATGTCAGTTGATTTAATTGCTTTTCTAAATT

pDA02_R      -----
pbpra2686_R  CACAAATGCATCAAGAGTAGCTTTAGCTTCTAAGTTTAGATCAATAGAATCATTTTCTTG

pDA02_R      -----
pbpra2686_R  CTGGTAATTATTTAACTTATTTTCTGAGATTACCAAATCTTTTTAATGCTTGGTAAATG

pDA02_R      -----
pbpra2686_R  CTCTTTTAAAAAAACCAAACCTATTTTCAGCTTCAGCCGAACCTCTCTCTACGTTTTGTAA

pDA02_R      -----
pbpra2686_R  TAGATAATCATGATTAATGCTATTTAAAATGCTTTCATTTTGATTTTATTACTACCAAT

```

Figure 83: Reverse sequence alignment of *pbpra2686* and pDA02

Clustalw and Boxshade alignment of *P. profundum* SS9 *pbpra2686* and pDA02. Blue box indicates the Stop site.

Appendix D– Cloning, expression and purification of *P. profundum* SS9 Ldh

D.1 Introduction

Along with dehydrogenases, Lactate dehydrogenase (Ldh) remains among the first enzymes to for examining protein biochemical adaptation to extreme environments, mostly because a large amount of kinetic data exists on dehydrogenases and there is a body of work accumulating comparing homologous proteins from mesophilic and psychrophilic backgrounds (Welch and Bartlett, 1997).

Respiratory enzymes catalyse the oxidation of respiratory substrates and pass the freed electrons to a variety of oxidants. The *P. profundum* SS9 putative Ldh encoded by *pbpra1210* shares significant homology to an *E. coli* fermentative Ldh (83% similarity). The fermentative Ldh takes an electron from NADH to catalyse the conversion of pyruvate to lactate.

This chapter describes the cloning and expression of the putative D-lactate dehydrogenase encoded by *pbpra1210* from *P. profundum* SS9R.

D.2 Cloning and expression of *P. profundum* SS9 Ldh in *E. coli* M15 pREP4 pQE30

The *P. profundum* SS9 gene *pbpra1210* is 1025 bp in size. Using the primers *pbpra1210*-F_SacI and *pbpra1210*-R_XmaI *pbpra1210* was amplified by PCR (Figure 84). This resulted in a PCR product of approximately 1000 bp. The product was cleaned by QIAquick PCR cleanup kit.

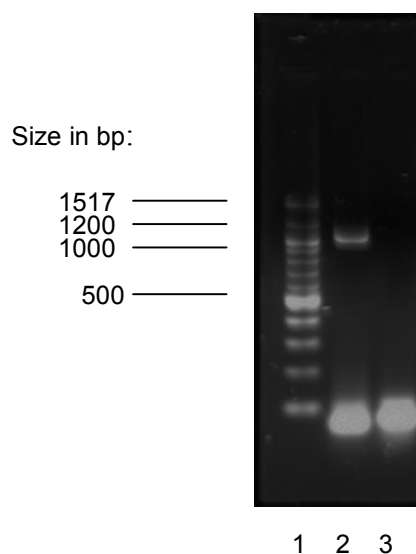


Figure 84: PCR of *pbpra1210*

The gene *pbpra1210* was amplified by PCR using the primers *Pbpra1210*-F_SacI and *Pbpra1210*-R_XmaI and separated on 1.3% w/v agarose gel. 100bp DNA ladder (NEB) (1), *pbpra1210* (2), *pbpra1210* ⁻ve, no template DNA (3). 61.8°C annealing temperature and 1 minute 30 seconds extension.

The *pbpra1210* PCR product was double digested using *SacI* and *XmaI* (Figure 85, lane 7). The vector pQE30 was also digested with both enzymes (Figure 85, lane 5). The products were then used in a ligation of the double digested gene and vector. After overnight ligation at 4°C the ligation mix was added to 25 µl competent *E. coli* M15 pREP4 and a basic transformation procedure was followed. By using pQE30 the expressed protein will have an N-terminal His tag added to simplify detection and selection.

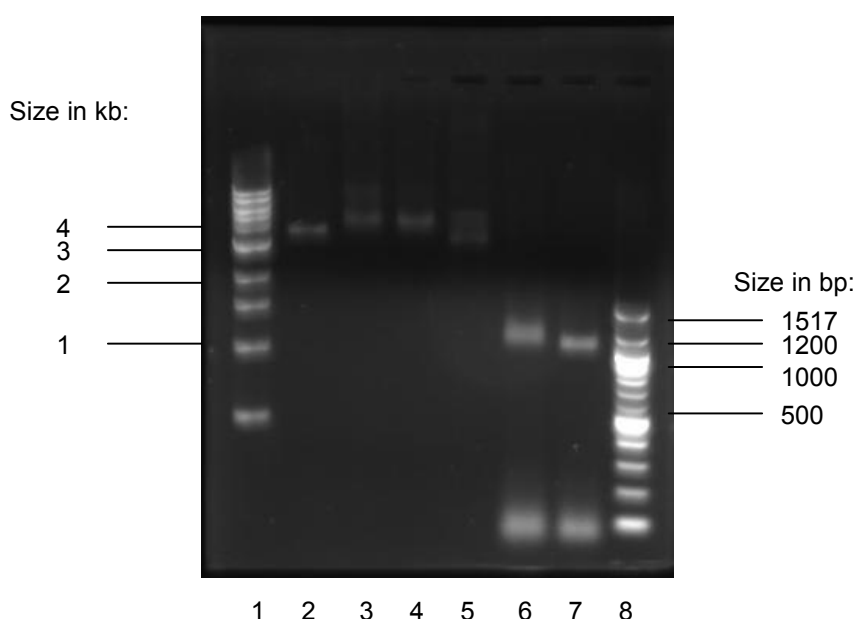


Figure 85: Restriction digest of pQE30Xa and *pbpra1210* PCR product

Both plasmid vector and PCR were double digested using *SacI* and *XmaI* and separated on 1.3% w/v agarose gel. 1 Kb DNA ladder (NEB) (1), pQE30 (2), pQE30 *SacI* digested (3), pQE30 *XmaI* digested (4), pQE30 double digested (5), *pbpra1210* PCR (6), *pbpra1210* PCR double digested (7), 100 bp DNA ladder (NEB) (8).

The transformants were purified twice on LB agar with 50 $\mu\text{g.ml}^{-1}$ kanamycin and 100 $\mu\text{g.ml}^{-1}$ ampicillin. Clones were screened by PCR and the majority were found to contain and insert of the correct size (Figure 86). *P. profundum* SS9R colony PCR was used as a positive control. A negative control was also included to demonstrate that an amplification could not be found from the *E. coli* M15 pREP4 containing the empty vector pQE30.

Induction experiments with these clones, however, were not always successful in detecting the His-tagged Ldh. In order to isolate clones that could be successfully induced using IPTG, a colony blot procedure was followed. This would screen the clones based on the detection of His-tagged Ldh.

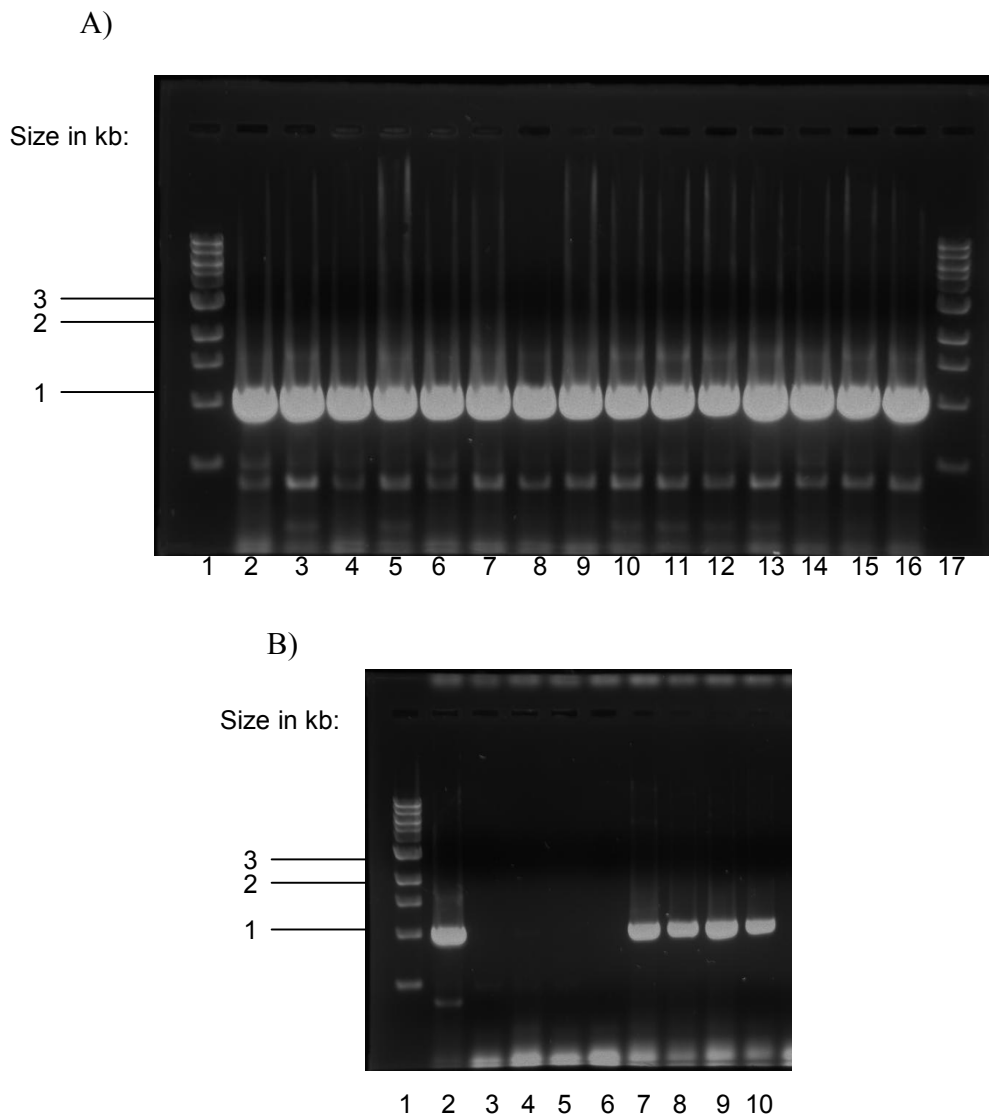


Figure 86: Detection of *E. coli* M15 pREP4 pQE30 + *pbpra1210* transformants by PCR

4 transformants were screened by PCR using the primers Pbpra1210-F_SacI and Pbpra1210-R_XmaI and, separated on 1% w/v agarose gel. The following temperature annealing gradient was used for each clone, 60.5°C, 61.8°C, 63.1°C, 64.2°C. (A) 1Kb DNA ladder (NEB), clone 1: (lanes 2, 3, 4 and 5), clone 2 (lanes 6, 7, 8 and 9), clone 3 (lanes 10, 11, 12 and 13), clone 4 (lanes 14, 15 and 16). B) 1 Kb DNA ladder (NEB) (1), clone 4 (2), *E. coli* M15 pREP4 pQE30 ^{-ve} control (lanes 3, 4, 5 and 6), SS9R positive control 60.5°C (lanes 7, 8, 9 and 10). 1 minute 30 seconds extension time.

The most efficient method of screening the transformants was to use an anti-His HRP conjugate and screen the transformants, not only for the presence of the *pbpra1210* insert, but also for the level of His-tagged protein production. The clones were purified and inoculated in spots in an LB agar plate containing the relevant antibiotics overnight. These were then transferred onto a nitrocellulose membrane and incubated on a fresh LB agar plate containing 1 mM IPTG. After induction the clones on the nitrocellulose membrane were lysed and screened with anti-His HRP (Figure 88).

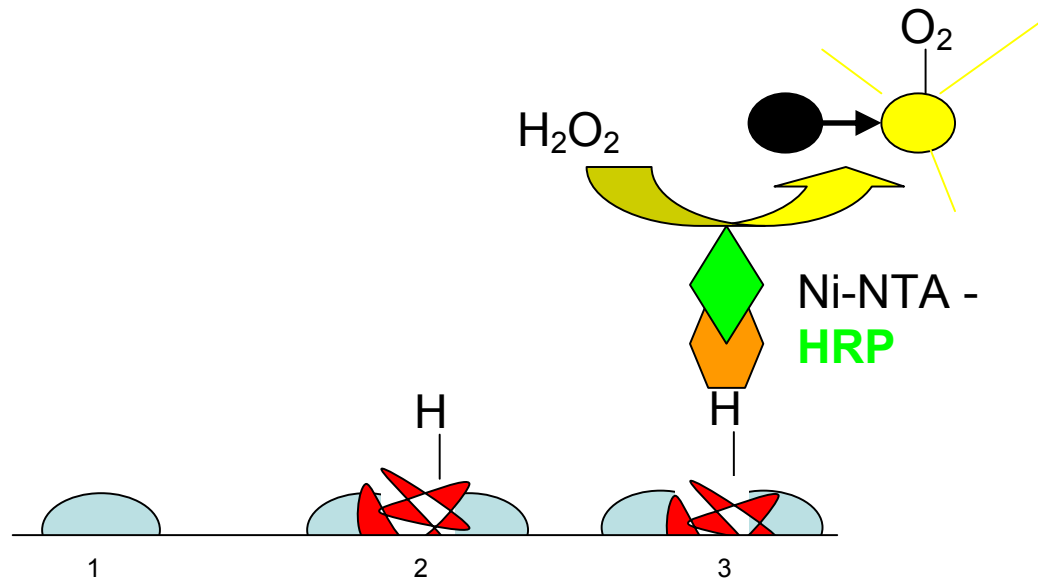


Figure 87: Basis of the colony blot procedure

1) Bacterial cells (blue semi-circle) were grown on LB agar and transferred to a nitrocellulose membrane. On the membrane they were induced with IPTG. 2) Cells were lysed and the His-tagged protein was exposed. (The red shapes represent protein and the 'H' is a His-tag). 3) The His-tagged proteins were detected by a Ni-NTA-Horse Raddish Peroxidase conjugate (orange-green shapes). Once in the presence of a suitable substrate light will be released through chemiluminescence.

Screening by colony blot was performed as described previously. The following example shows the positive and negative controls used in the screen (Figure 88, 3 and 4 respectively). The positive control was an *E. coli* strain containing a known His-tagged protein, in this case CysK from *Salmonella*. The negative control was an *E. coli* strain containing the empty expression vector pQE30. The screen was performed in duplicate, with 4 replicate colonies (Figure 88, 1C and 2C). On the duplicate nitrocellulose membranes were also negative controls (Figure 88, 1 and 2, A and B). In this screen it can be seen that of the 4 potentially positive clones, the fourth gives the strongest signal. The fourth clone (furthest right in C) was therefore used for subsequent induction and purification experiments. The plasmid was purified and named pDA05.

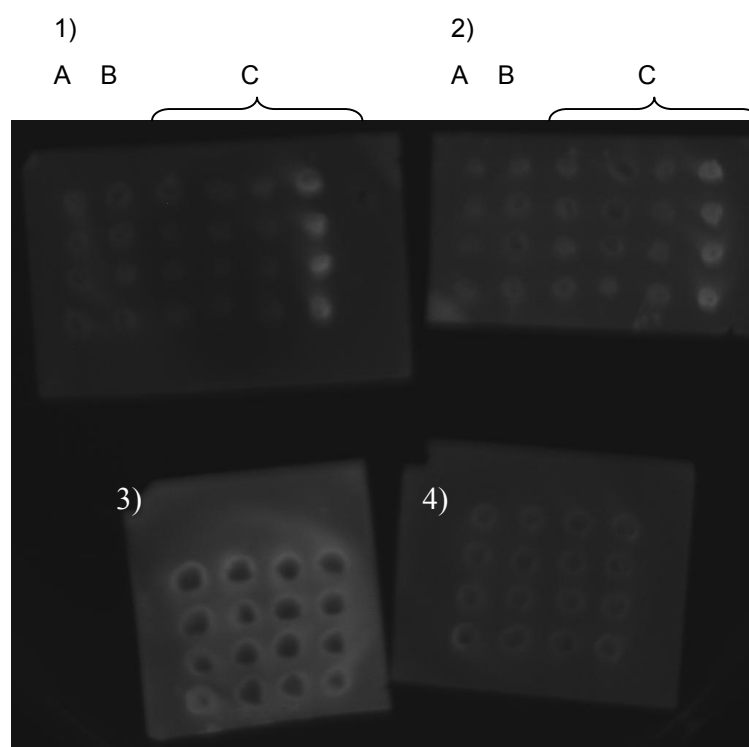


Figure 88: Detection of *E. coli* M15 pREP4 pDA05 transformants by colony blot

1) Screen 4 possible positive strains, 2) repeat of screen, 3) *E. coli* BL21 231A pETDEST42::cysK-His positive control, 4) *E. coli* M15 pREP4 pQE30, negative control. A) *E. coli* M15 pREP4 pFL202, B) *E. coli* M15 pREP4 pQE30Xa, C) 4 possible pDA05 positive strains, repeated 4 times.

To assess the effect of induction with IPTG on cell growth, cultures with pQE30 or pDA05 were induced with and without 1 mM IPTG and the OD₆₀₀ was monitored over time (Figure 89). After approximately 2 hours growth at 37°C and 200 rpm, 1 mM IPTG was added. The graph demonstrates that the addition of IPTG did not significantly affect the growth of *E. coli*, and that the production of Ldh from the induction did also not significantly affect growth. In fact, *E. coli* with pDA05 and + IPTG had a greater exponential growth rate and high final OD₆₀₀ than the 2 controls (compare Figure 89 ♦ with ■ and □).

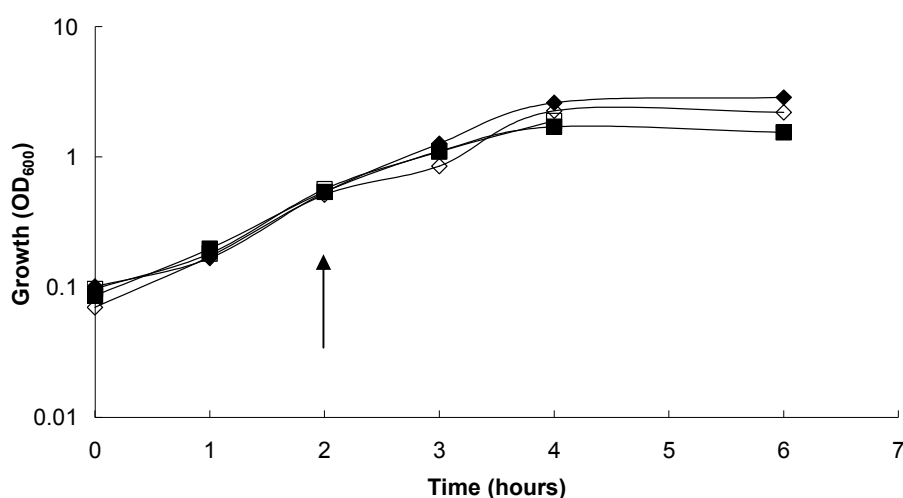


Figure 89: Induction of Ldh expression by addition of IPTG

Stationary phase cultures of *E. coli* M15 pREP4 pQE30 + *pbpra1210* (pDA05) and *E. coli* M15 pREP4 pQE30Xa were diluted to an optical density of 0.1 and incubated at 37°C 200 rpm until the OD₆₀₀ was between 0.4 and 0.6. At this point (2 hours) 1mM IPTG was added to induce expression (indicated by arrow). The OD was monitored to ensure the addition of IPTG was not detrimental to the growth of the culture. (■) *E. coli* M15 pREP4 pQE30 + IPTG, (□) *E. coli* M15 pREP4 pQE30 - IPTG, (♦) *E. coli* M15 pREP4 pDA05 + IPTG, (◇) *E. coli* M15 pREP4 pDA05 - IPTG.

D.3 Purification of *P. profundum* SS9 Ldh

Before purification, the solubility of the protein must be assessed. To determine the Ldh protein solubility, a 5 ml culture was induced to express Ldh using 1 mM IPTG for 3 hours before being pelleted. The pellet was resuspended in lysis buffer (see methods 2.10.12) and 1mg.ml⁻¹ lysozyme was added and the solution was sonicated at 10 amplitude microns for 10 seconds with 10 second pauses, 6 times in total. The solution was then centrifuged at 9000 x g for 30 minutes to separate the soluble and insoluble phases, then analysed by SDS-PAGE. The majority of the induced protein (predicted size 38 kDa) was found to be in the insoluble phase (Figure 90). The 2 bands seen arise most likely a product of protease breakdown due to the fact that only one of the bands appears to be his-tagged. The western blot shown in B also shows that the induced band is also His tagged, further suggesting that this band corresponds to the induced Ldh.

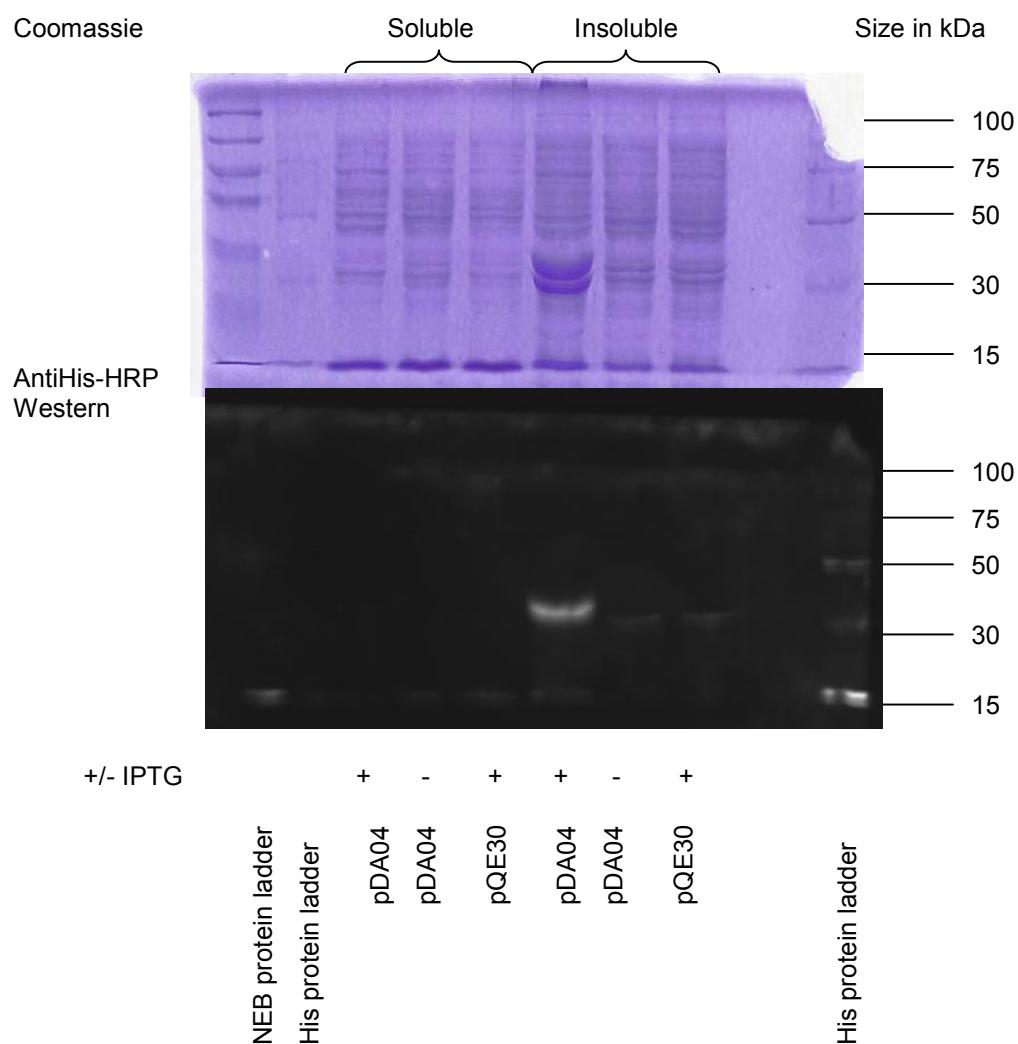


Figure 90: Induction of Ldh expression by addition of IPTG

E. coli pREP4 with pDA04 or pQE30 were induced with 1 mM IPTG for 3 hours. The cultures were pelleted, sonicated and separated into soluble and insoluble phases before being analysed by SDS-PAGE with either coomassie staining or western blotting with Qiagen Ni-NTA HRP.

For the purification of Ldh it required the protein to be mostly soluble. The simplest method to solubilise the Ldh was to alter the induction parameters, such as time, IPTG concentration, temperature, etc. The first attempt to solubilise the Ldh was performed by inducing the cells for various periods. However, the band of the induced protein at 38 kDa can still be clearly seen in the insoluble phase mostly after 2 or 3 hours induction (Figure 91). There is however a small signal in the soluble phase after 3 hours.

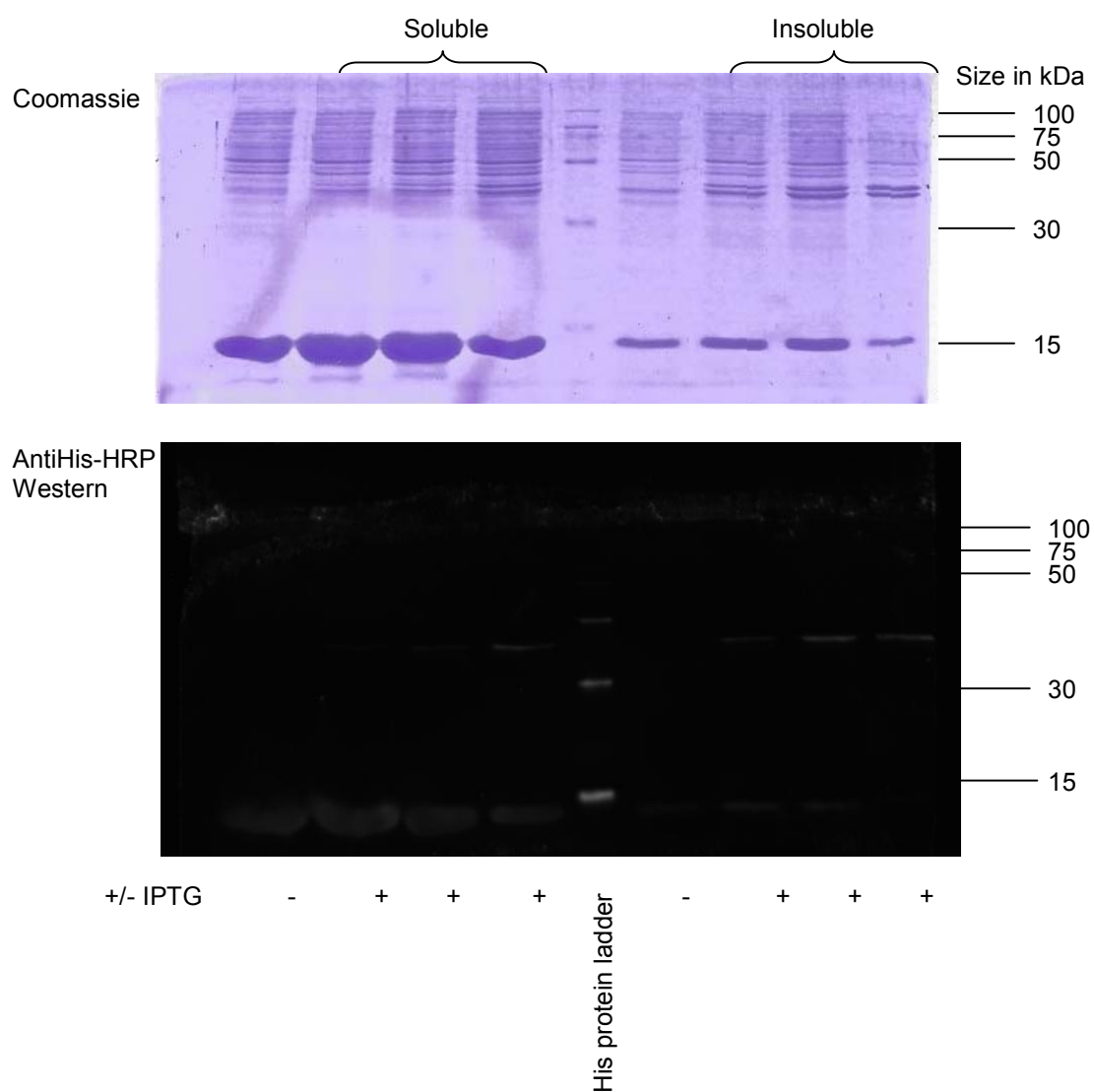


Figure 91: Induction of Ldh expression by addition of IPTG at various times

E. coli pREP4 with pDA04 was induced with 1 mM IPTG for 1, 2 and 3 hours. The cultures were pelleted, sonicated and separated into soluble and insoluble phases before being analysed by SDS-PAGE with either coomassie staining or western blotting with Qiagen Ni-NTA HRP.

The next experiment to try to increase the solubility of the Ldh was to reduce the amount of IPTG used for the induction and also to reduce the induction temperature. This involved using 1, 0.1 and 0.01 mM IPTG and 1 mM IPTG at 18°C overnight (Figure 92). Induction at 37°C with 1 mM and 0.1 mM IPTG still resulted in the majority of Ldh being insoluble. Reducing the IPTG concentration to 0.01 mM had no obvious effect on Ldh solubility, but did reduce the overall level of expression. Induction overnight at 18°C with 1 mM IPTG also resulted in the majority of Ldh still being insoluble.

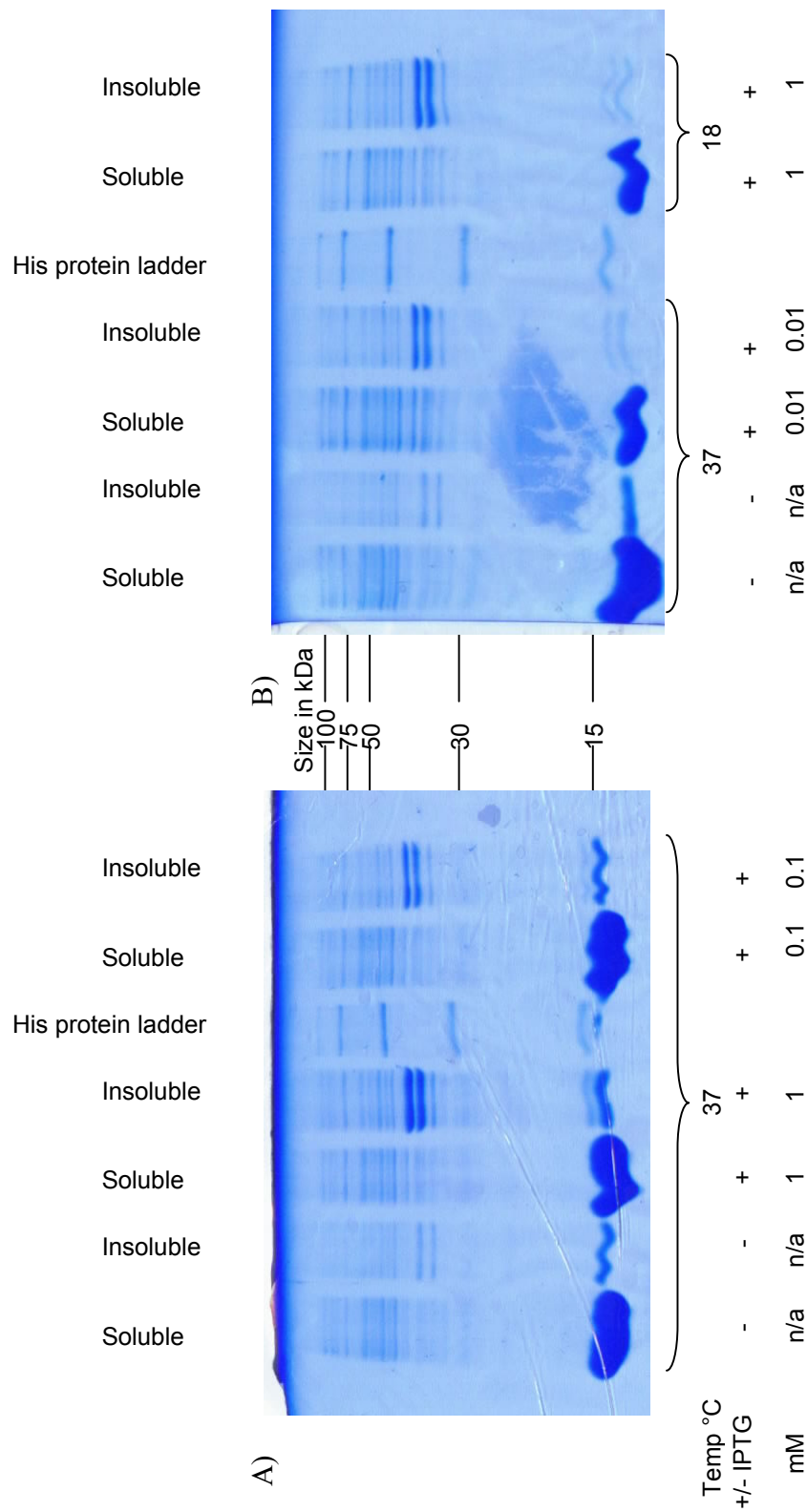


Figure 92: Induction of Ldh expression into soluble phase by reduction of IPTG and temperature

E. coli M15 pREP4 pDA05 was induced for 3 hours as described, then cultures were sonicated and separated into soluble and insoluble phases as described previously. Proteins were analysed by SDS-PAGE and Coomassie stained.

Due to apparent insolubility of the *P. profundum* SS9 Ldh, it was decided that the Ldh would be purified under denaturing conditions. This would allow the protein to be solubilised, however the activity of the protein would most likely be affected. After purification under such conditions, the protein must be refolded. The purification was accomplished by resuspending the insoluble pellet from a 3 hour 37°C induction with 1 mM IPTG in an 8M Urea solution. This was left at room temperature for 1 hour, then separated again as before into soluble and insoluble phases. This demonstrated that the large amount of insoluble protein can be made soluble by the urea treatment (Figure 93).

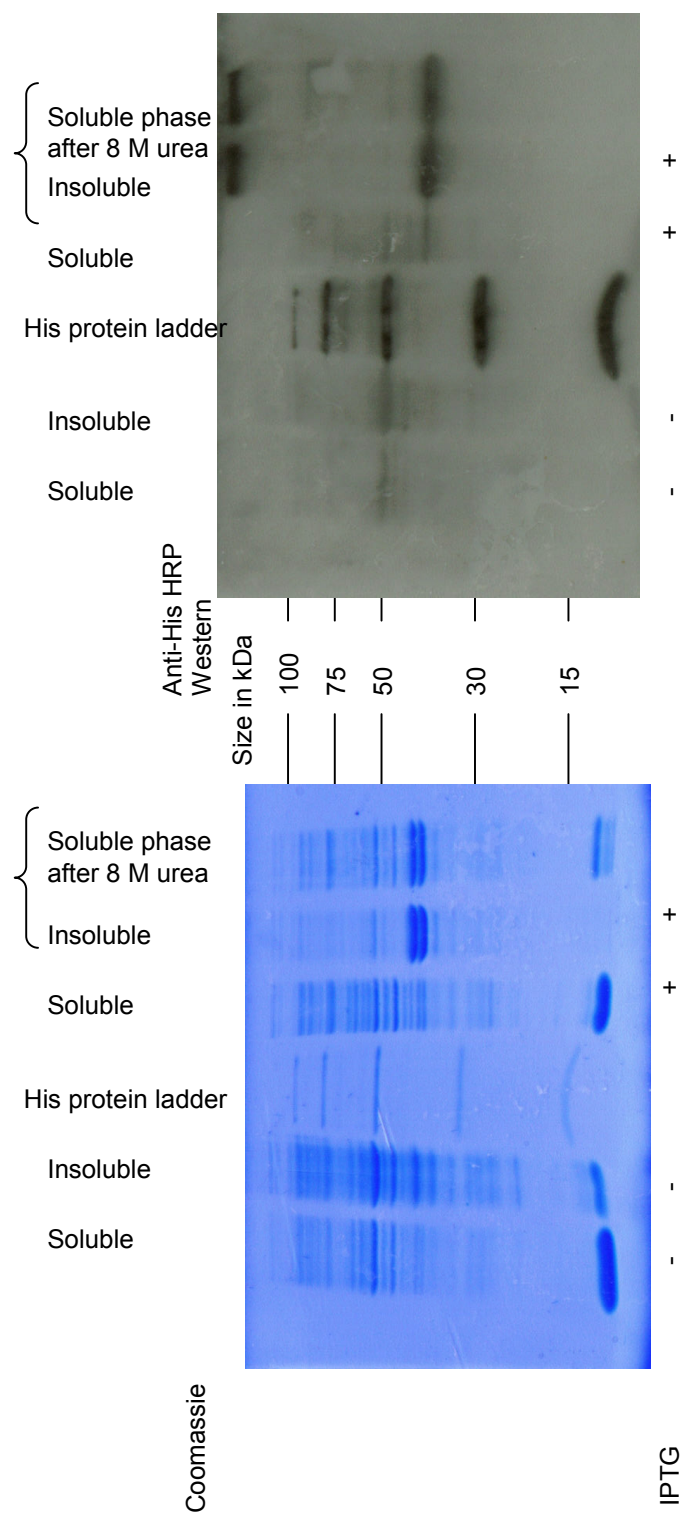


Figure 93: Solubilisation of Ldh using 8 M Urea

A 3 hour, 37°C induction using 1 mM IPTG was sonicated and separated into soluble and insoluble phases as previously described. The insoluble pellet was then resuspended in an 8 M urea solution for 1 hour. This suspension was then separated again before being analysed by SDS-PAGE and A) Coomassie stained and B) Western blotted probed with anti-His HRP.

E. coli M15 pREP4 pDA05 were induced with 1 mM IPTG for 3 hours at 37°C, then separated into soluble and insoluble phases, with the insoluble phase having the Urea treatment as previous described. The His-tagged Ldh was then purified using Qiagen purification columns and a Ni-NTA agarose mixture (described fully in methods 2.10.12). By eluting with a buffer pH 6.3 a significant amount of His-tagged protein was able to be purified (Figure 94).

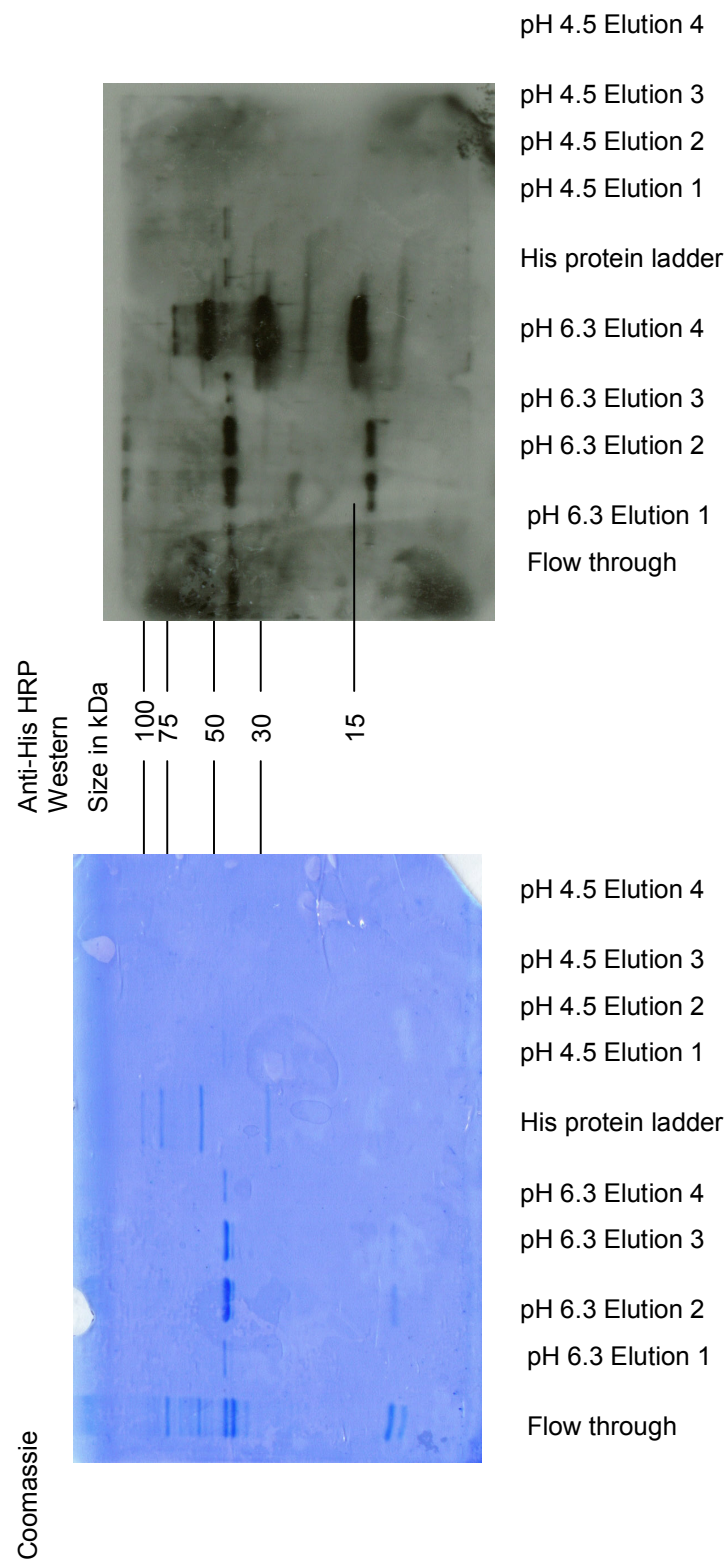


Figure 94: Purification of *P. profundum* SS9 His-tagged Ldh using Ni-NTA agarose

Induction was sonicated and separated into soluble and insoluble phases by the denaturing purification protocol. The soluble phase from the denaturing purification protocol was then passed through 4 columns with Ni-NTA agarose and eluted with either elution buffer of either pH 6.3 or 4.5. The elutant was then analysed by SDS-PAGE and A) Coomassie stained and B) Western blotted and probed with anti-His HRP.

D.4 Discussion

D.4.1 Cloning and screening of pbpra1210

The *P. profundum* SS9 gene *pbpra1210* encoding a putative D-lactate dehydrogenase was successfully cloned into pQE30. While screening clones by PCR and restriction digest is commonly the quickest and simplest method of identifying clones, this was not completely suitable for this study. In this case, a colony blot procedure was adopted, in which individual colonies were induced by addition of IPTG, lysed and probed with Ni-NTA HRP all on a nitrocellulose membrane. This allowed those clones with high levels of expression to be selected.

D.4.2 Expression and purification of *P. profundum* SS9 Ldh

Expression of the *P. profundum* SS9 Ldh was found to result in largely insoluble protein, which is difficult to purify. In order to enable purification various methods were employed to solubilise the protein, most of which concentrated on modification of the induction conditions. Often insoluble proteins are in the insoluble phase due to an overexpression, by reducing this level of expression the protein can sometimes be made soluble. In this case the concentration of IPTG was reduced, the length of induction was reduced and an overnight expression at 18°C was also tried. In all cases no significant effect on the solubility of the Ldh was found. Therefore, in order to solubilise the protein denaturing conditions were used. By resuspending the insoluble phase in an 8 M urea solution and separating by centrifugation, a large amount of the previously insoluble protein was made soluble. This consisted of a band at approx 38 kDa which represented the *P. profundum* SS9 Ldh. The now soluble Ldh was then purified using Ni-NTA agarose to bind the His-tagged protein. The protein was found to elute best in the presence of an elution buffer at pH 6.3.

D.4.3 Conclusions

While the *P. profundum* SS9 Ldh was successfully cloned and shown to be capable of purification, further experiments on the Ldh activity and kinetics are still required. Before these can be performed the urea must be diluted out and the protein refolded. Unfortunately, no further work was performed on the Ldh activity due to time limitations.

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